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VOLUME 98, JANUARY-JUNE, 1956

TABLE OF CONTENTS

No. 1. January–February, 1956

PAGE

Jesús Kumate, Lázaro Benavides V., José L. Pérez N., Oscar Criollos T., and Julio Carrillo. Pathological physiology of salmonellosis. II. Plasma cholinesterase in infections due to Enterobacteriaceae 1

D. D. Stamm, R. E. Kissling, and M. E. Eidson. Experimental rabies infection in insectivorous bats 10

J. B. Macdonald, R. M. Sutton, M. L. Knoll, E. M. Madlener, and R. M. Grainger. The pathogenic components of an experimental fusospirochetal infection 15

L. Joe Berry. Susceptibility to infection as influenced by acclimatization to altitude and Krebs cycle inhibitors and intermediates 21

Jacqueline Baumeister and C. Arden Miller. The preparation of poliomyelitis complement-fixing antigens from infected tissue culture fluids ... 27

J. van der Hoeden. Leptospirosis canicolaris in pigs and its probable transfer to human beings 33

Coit M. Coker. Some effects of cortisone in mice with acquired immunity to *Trichinella spiralis* 39

Erela Ephrati-Elizur and Hans Bernkopf. Isolation of six strains of ornithosis virus from children with infections of the respiratory tract 45

J. A. Morris, C. G. Aulisio, and J. M. McCown. Serological evidence of toxoplasmosis in animals 52

Joseph Victor, Dorothy G. Smith, and Abou D. Pollack. The comparative pathology of Venezuelan equine encephalomyelitis 55

Willy Burgdorfer and Cora Rust Owen. Experimental studies on argasid ticks as possible vectors of tularemia 67

Bernard N. Jaroslow and William H. Taliaferro. The restoration of hemolysin-forming capacity in X-irradiated rabbits by tissue and yeast preparations 75

Doki Chun and Bohan Park. Demonstration of *Shigella flexneri* antigens by means of hemagglutination test 82

Susumu Hotta and Charles A. Evans. Cultivation of mouse-adapted dengue virus (type 1) in rhesus monkey tissue culture 88

J. M. Vanella, R. E. Kissling, and R. W. Chamberlain. Transmission studies with encephalomyocarditis virus 98

D. C. Cavanaugh, C. M. Wheeler, W. Suyemoto, Tomiko Shimada, and Yoko Yamakawa. Studies on *Pasteurella pestis* in various flea species. I. Infection of flea species with avirulent strains of *Past. pestis* 103

C. M. Wheeler, W. Suyemoto, D. C. Cavanaugh, Tomiko Shimada, and Yoko Yamakawa. Studies on *Pasteurella pestis* in various flea species. II. Simplified method for the experimental infection of fleas 107

No. 2. March–April, 1956

George J. Scheff, Irene M. Scheff, and Gilbert Eiseman. Concerning the mechanism of bartonella anemia in splenectomized rats 113

R. H. Roberts, R. J. Dicke, R. P. Hanson, and D. H. Ferris. Potential insect vectors of vesicular stomatitis in Wisconsin 121

Francis W. Hughes and Arthur L. Tatum. Effects of hypoxia on rats infected by *Trypanosoma lewisi* 127

PAGE

J. R. M. Innes, Catherine Wilson, and Martin A. Ross. Epizootic *Salmonella enteritidis* infection causing septic pulmonary phlebothrombosis in hamsters 133
P. G. Pande and D. Krishnamurty. Paratido-stomatitis in calves 142
W. D. Winter, Jr. and G. E. Foley. Enhancement of candida infection: differential distribution of renal lesions in mice treated with aureomycin 150
G. J. Thorbecke and F. J. Keuning. Antibody and γ globulin formation in vitro in hemopoietic organs 157
Lois Norman, A. W. Donaldson, and E. H. Sadun. The flocculation test with a purified antigen in the diagnosis of trichinosis in humans 172
Edward Katz. The activity of tetracycline on feline pneumonitis virus infection of chick embryos 177
Coit M. Coker. Cellular factors in acquired immunity to *Trichinella spiralis*, as indicated by cortisone treatment of mice 187
L. Joe Berry, Madeleine K. De Ropp, Marjory H. Fair, and Eva M. Schur. Dynamics of bacterial infections in mice under conditions known to alter survival time 198
L. L. Uzman, H. Rosen, and G. E. Foley. Studies on the biology of *Cryptococcus*. VI. Amino acid composition of the somatic proteins of *Cryptococcus neoformans* 208
Lowell A. Rantz, Elizabeth Randall, and Anne Zuckerman. Hemolysis and hemagglutination by normal and immune serums of erythrocytes treated with a nonspecies specific bacterial substance 211

No. 3. May–June, 1956

Richard F. Berendt and Walter J. Nungester. The effect of nasal secretions upon human serum 223
James W. Moulder, Julio I. Colón, Jacqueline Ruda, and Marian M. Zebovitz. The effect of penicillin on multiplying and nonmultiplying populations of sensitive and resistant strains of feline pneumonitis virus 229
Monroe D. Eaton, Louise T. Adler, Phyllis Bond, and A. R. Scala. The effect of thyroxin on respiration and virus growth in chick embryo tissue cultures 239
Donald G. Low, C. W. Hiatt, Chester A. Gleiser, and E. N. Bergman. Experimental canine leptospirosis. I. *Leptospira icterohemorrhagiae* infections in immature dogs 249
Donald G. Low, E. N. Bergman, C. W. Hiatt, and Chester A. Gleiser. Experimental canine leptospirosis. II. Renal function studies 260
B. K. Nordberg and K. Schjerning-Thiesen. Detection of complement fixing antibodies against foot-and-mouth disease in cattle serum 266
Hilton B. Levy and Leroy F. Snellbaker. Effects of animal viruses on host cell metabolism. I. Effects of encephalomyocarditis virus on nucleic acids and phosphorus fractions of Ehrlich ascites tumor cells 270
David W. Talmage and Gloria G. Freter. The blocking of labeled antibody uptake as a measure of the relative combining capacity of anti-red cell serums 277
David W. Talmage, Gloria G. Freter, and William H. Taliaferro. The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums 293
David W. Talmage, Gloria G. Freter, and William H. Taliaferro. Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation 300
J. A. Morris, J. M. McCown, and R. E. Blount. Ascites and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures 306
D. Pappagianis, C. E. Smith, and G. S. Kobayashi. Relationship of the in vivo form of *Coccidioides immitis* to virulence 312
Index 320

J. Infect. Dis. Vol. 98, No. 3

THE EFFECT OF NASAL SECRETIONS UPON HUMAN SERUM

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The discovery of the resistance-lowering properties of hog gastric mucin (Nungester et al, 1932) has been followed by a number of studies on the properties of mucins lowering host resistance. Although the great majority of these studies have dealt with the production of infection in experimental animals, a number of workers have reported the alteration of the antibacterial properties of the blood following treatment with hog gastric mucin (Nungester et al, 1936; Keefer and Spink, 1938; Oerskov, 1940; Fothergill et al, 1937; Engley, 1954a and b).

Although the mechanisms of the antibacterial effects of blood have not been fully described, the importance of serum complement has repeatedly been emphasized particularly as affecting the gram-negative organisms. An important antibacterial phenomenon which involves complement has recently been described by Pillemer (1954), who has reported the isolation of an antibacterial serum protein, properdin, which acts upon susceptible organisms in a complement-dependent reaction.

The anticomplementary activity of hog gastric mucin has been reported by Lambert and Richley (1952). Also, the characterization of one of the active constituents of hog mucin as a heparin by Smith and co-workers (1952) provides an explanation of the mechanism of this reaction in view of the earlier reports of the anticomplementary activity of heparins (Ecker and Gross, 1929; Gross and Ecker, 1929; Wadworth et al, 1937). Certain antibacterial phenomena, however, are due to relatively thermostable serum components, β-lysins, and do not involve complement. The inactivation of β-lysin by hog gastric mucin has been reported by Engley (1954b).

In this study the effect of human nasal secretions upon serum complement and its components and β-lysin has been studied in an attempt to determine whether unphysiological accumulations of upper respiratory mucus might have deleterious effects upon the host.

METHODS AND MATERIALS

Nasal secretions.—Nasal secretions samples were obtained from two sources: saline irrigation of the nasal passages of normal individuals and irrigation of the maxillary sinuses of persons believed to have sinusitis.*

In the initial studies the samples, regardless of of source, were pooled, dialysed against distilled water at 4 C until free from chlorides and then lyophilized. The dried material was then suspended at a convenient concentration, usually 10 to 20 mg per ml in 0.01 *M* phosphate buffer, pH 7.2, and sterilized by autoclaving at 121 C for 3 minutes.

Serum.—Normal human serum was obtained by venipuncture of apparently normal donors. The blood was allowed to clot, centrifuged to remove formed elements, and the serum was distributed in 5.0 ml aliquots in sterile screw-capped tubes. The tubes were then placed in a deep-freeze at approximately −60 C and were removed as needed.

Complement titrations.—Complement titrations were carried out by the method of Kabat and Mayer (1948), except that a 2% sheep cell sus-

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pension was substituted for the 5% preparation recommended by these workers.

The effect of the nasal secretions upon the individual components of complement was determined with specifically inactivated serums according to the procedures of Ecker et al (1943). However, the effect of the secretions upon the first and second components was determined by preparing serums with both the first and second components inactivated (H). This was done by heating a sample of serum for 15 minutes at 56 C.

The zymosan which was used in the preparation of Z was made from fresh bakers' yeast by the method of Pillemer and Ecker (1941).

Estimation of antibacterial activity.—The antibacterial activity of the various serum preparations was determined by a modification of the method of Eckstedt (1954). This technique consists of adding the desired number of organisms (suspended in sterile saline) to a given volume of serum, stoppering the tubes, incubating at 37 C for 6 hours, and finally determining the organism concentration of a 1:1000 saline dilution of the preparation by standard pour-plate techniques.

Test organism preparations.—The organism selected was a strain of *Streptococcus pyogenes*, group A, type 3, Richard's strain. The streptococci were cultured at 37 C for 24 hours in brain-heart infusion broth (Difco) and stored at approximately −60 C in a deep freeze until needed. For use the thawed culture was transferred to the same medium and incubated at 37 C for 16 hours. Population estimations showed approximately 5×10^6 organisms per ml at this time. After every 14 serial subcultures a new culture was used.

EXPERIMENTAL PROCEDURES AND RESULTS

Initially, the effect of the secretions upon the hemolytic complement titer of serum was determined. For this purpose a doubling dilution series of secretions, ranging in concentration from 5.0 to 1.56 mg per ml, was prepared in sterile buffer. Exactly 0.25 ml of each dilution of the secretions was then added to 0.5 ml of serum, the tubes were stoppered, incubated at 37 C for 60 minutes, and the complement titer of each sample determined. The results of these tests (table 1) demonstrated a reduction in complement titer which was roughly proportional to the concentration of secretions in the mixture.

TABLE 1.—*The effect of nasal secretions upon the complement titer of normal human serum.*

Concentration of nasal secretions (mg per ml of serum)	Complement titer (units per ml of serum)
2.5	0
1.25	80
0.625	80
0.3125	100
0.156	133
0.078	160
0.0 (control)	160

To explain the alteration of the complement titer a series of studies were run to determine which of the four known components of complement were affected.

Effect on C'_1 and C'_2.—The effect of the secretions upon human C'_1 and C'_2 was determined by titrating secretion-treated serum with H. The results (table 2) indicated that the first and second components were unaffected. However, the interpretation of results was complicated by the observation that the mixture of treated serum and H had a titer of only 100 units. Further study using Z and N (table 2) showed that the lowered titers were due to the partial inactivation of C'_3 and C'_4 in the H preparations.

Effect on C'_3 and C'_4.—The effect of nasal secretions upon the third and fourth components was determined in a manner similar to that previously described for the first and second components. The secretion-treated serum was

TABLE 2.—*The effect of nasal secretions upon the first and second components of serum complement.*

Solution tested	Complement titer (units per ml of serum)
Normal serum control	160
H*	0
Z†	0
N‡	0
Nasal secretion-treated serum	0
Nasal secretion-treated serum plus H	100
H plus Z	100
H plus N	100

* Serum lacking the first and second components of complement.
† Serum lacking the third component of complement.
‡ Serum lacking the fourth component of complement.

TABLE 3.—*The effect of nasal secretions upon the third and fourth components of serum complement.*

Solution tested	Concentration of nasal secretions (mg/ml of serum)	Complement titer (units/ml of serum) titrated with	
		Z	N
Serum plus buffer		160	160
Untreated serum		160	160
Z*		0	160
N†		160	0
Treated serum	10.0	0	0
Treated serum	5.0	0	80
Treated serum	2.5	0	100
Treated serum	1.25	80	133
Treated serum	0.625	100	160
Treated serum	0.3125	133	160
Treated serum	0.156	100	160

* Serum treated with zymosan to inactivate C'_3.
† Serum treated with ammonia to inactivate C'_4.

titrated for C'_3 with Z and for C'_4 with N. The results (table 3) indicated total destruction of C'_3 with concentrations of secretions as low as 2.5 mg per ml of serum and a lesser effect upon the fourth component.

Effect of nasal secretions on β-lysin.—Initially it was necessary to determine the sensitivity of the streptococci to the serum preparations which were employed, and also to demonstrate the independence of the reactions from complement. For this purpose 0.25 ml of a 10^{-6} dilution of the organism was added to 0.5 ml of serum which previously had been heated for 30 minutes at 56 C. This suspension was then tested for antibacterial activity, and the results were compared with those obtained with untreated serum. In addition, the complement titer of both preparations was determined. The results demonstrated no change in the antibacterial activity of the serum although the heat-treatment was sufficient to destroy all complementary activity. In order to avoid complications which might be due to complement, heated serum was used in subsequent experiments.

In order to determine the effect of nasal secretions upon the above reaction a series of 10-fold dilutions of pooled secretions, containing from 5.0 to 0.0005 mg per ml, was prepared. Prior to the addition of the test organism suspension, 0.25 ml of secretions was added to 0.5 ml of serum and incubated at 37 C for 60 minutes. In a control tube the secretions were replaced by buffer. The organism suspension was then added to each tube and the incubation continued for 6 hours. The results of a typical study (table 4) demonstrated reduction in antibacterial activity which was proportional to the concentration of the nasal secretions employed.

Effect of alkaline shift.—In the course of experimentation to determine the mechanism of the reactions which have been noted above, two possible explanations for the activity of the nasal secretions were postulated. The first of these was that the secretions combined in some sort of direct chemical reaction with the β-lysin. The second was based upon the findings of Fuller and coworkers (1939) and Hench (1952), who have claimed that the killing of hemolytic streptococci and pneumococci respectively by serum in vitro is the result of an alkaline shift in the pH of the serum, due to carbon dioxide loss, and is not solely the result of an intrinsic germicidal factor of the serum. If the alkaline shift of the serum was taken as the basis of the germicidal activity, then the activity of the nasal secretions in preventing the germicidal action of serum could be best explained on the basis of a buffering action of the secretions.

In order to determine which of the two hypotheses provided the best ex-

TABLE 4.—*The effect of nasal secretions upon the β-lysins of normal human serum.*

Solution tested	Concentration of nasal secretions (mg/ml of serum)	Number of *Str. pyogenes* per ml, 6 hour count
Serum plus buffer	0.0	0
Serum plus secretions	2.5	1.2×10^7
Serum plus secretions	0.25	6.0×10^6
Serum plus secretions	0.025	2.0×10^5
Serum plus secretions	0.0025	1.0×10^5
Serum plus secretions	0.00025	0

planation for the observed action of nasal secretions on the antistreptococcal action of serum, two parallel series of serum samples, containing graded amounts of nasal secretions, were prepared. One series of tubes was closed with a rubber stopper and the other with a cotton plug. After 6 hours incubation the organism concentration and the pH of each tube was determined. The results of a typical experiment are given in table 5. They show that although a marked alkaline shift occurred in the tubes with cotton plugs and not in the rubber-stoppered tubes, the streptococci were unaffected by pH changes of the order of magnitude observed. In addition, the secretions apparently did not have any significant buffering ability.

Comparative studies.—In order to determine the extent of variation between individual samples of nasal secretions, as well as to determine whether infected sinus secretions possessed more activity than normal nasal specimens a comparative study was carried out. For this purpose 10 normal nasal and 10 infected sinus washings were individually dried and resuspended. Each sample was then tested for activity using an arbitrary system of grading activity. The end point of activity in respect to β-lysin for each sample was taken as that dilution in a series of tenfold dilutions, which facilitated the growth of ten times as many organisms as were present in the control plates. The end point of activity in respect to C'_3 and C'_4 was the minimal concentration giving complete inactivation of the component in question.

TABLE 5.—*The effect of the alkaline shift upon the anti-streptococcal activity of serum and the effect of nasal secretions upon the shift.*

Closure	Concentration of secretions (mg/ml of serum)	pH after 6 hours incubation	Number of *Str. pyogenes* per ml, 6 hour count
Cotton plug	0.0	8.7	0
	10.0	8.65	2×10^7
	1.0	9.0	5×10^8
	0.1	8.8	1×10^8
	0.01	9.0	0
	0.001	8.8	0
Stopper	0.0	7.7	0
	10.0	7.35	5×10^7
	1.0	7.3	2×10^8
	0.1	8.4	1×10^8
	0.01	7.8	1×10^8

TABLE 6.—*Comparison of the activity of individual samples of normal nasal and infected sinus specimens.*

Sample no.	Inactivation of serum factor (mg of secretions/ml of serum)		
	C'_3	C'_4	β-lysin
Normal nasal washings			
1	20.0	Inactive*	10.0
2	10.0	20.0	10.0
3	5.0	5.0	Inactive
4	20.0	Inactive	10.0
5	10.0	20.0	1.0
6	2.5	20.0	0.1
7	1.25	10.0	Inactive
8	5.0	10.0	10.0
9	2.5	10.0	10.0
10	5.0	10.0	10.0
Average	7.9	13.1	7.64
Infected sinus washings			
1	20.0	Inactive	10.0
2	5.0	20.0	1.0
3	20.0	Inactive	0.1
4	5.0	10.0	0.01
5	2.5	10.0	0.1
6	20.0	20.0	10.0
7	2.5	2.5	0.01
8	10.0	10.0	1.0
9	20.0	Inactive	10.0
10	5.0	20.0	0.1
Average	11.0	13.5	3.14

* Inactive at concentrations of 20.0 mg per ml or less. No tests were carried out at higher concentrations.

The results (table 6) indicated that although there was considerable variation between individual samples there was little difference in the average of results obtained with normal nasal and infected sinus specimens.

Effect of dialysis or autoclaving on secretions.—Although nasal secretions demonstrated marked activity against certain humoral factors, it was necessary to show that the samples neither gained nor lost activity in the process of preparation. Two of the preparatory steps were thought to be of importance: dialysis and autoclaving. Autoclaving of the secretions in particular was a critical step since Smith (1950) and Anderson and Oag (1939) have demonstrated a loss in the resistance lowering activity of hog gastric mucin following autoclaving.

For an investigation of the effect of preparatory techniques three criteria of activity were available: the destruction of the third and of the fourth components of complement, and the alteration of β-lysins.

The effect of autoclaving was determined by pooling a number of samples of lyophilized sinus specimens and suspending them in 0.01 *M* phosphate buffer at pH 7.2. One-half of the suspension was autoclaved at 121 C for 3 minutes and the remainder was used with no further treatment. Both samples were then tested for effect upon the various serum factors by the methods which have been described. The results indicated that the heat treatment caused no change in the activity of the secretions.

The possibility of alteration of activity following dialysis was investigated by pooling several samples of lyophilized secretions and suspending them in distilled water at a concentration of 20.0 mg per ml. An aliquot was removed for total nitrogen determination by the micro-Kjeldahl method (Kabat and Mayer, 1948), the balance was divided in half and the exact volume of each sample was determined. One sample was dialysed against distilled water at 4 C overnight, and the other was held at this temperature for the same period. Both samples were then lyophilized and resuspended in the original volume of buffer. The nitrogen content of each sample and the activity of each in respect to the inactivation of the three serum factors was then determined. The results showed that there was no loss in activity although approximately 20 per cent of the total nitrogen was lost upon dialysis.

DISCUSSION

The fundamental problem of this study was to determine whether nasal secretions possessed inhibitory activity against several humoral defense mechanisms. This activity has been clearly demonstrated by the experimental results. The anticomplementary activity of the secretions has been shown to be due to the inactivation of the third component although in some cases the fourth component was also affected. However, the third and fourth components are apparently not affected by the same constituents of the secretions. Proof of this can be found in the results of the comparative study (see table 6) in which no correlation could be found between anti-third and anti-fourth component activity.

The experimental data also show that nasal secretions are capable of altering the action of β-lysins. The mechanism of this alteration was not determined, but the experimental evidence points to a direct reaction between the secretions and some component of the β-lysin system.

A more significant observation, demonstrated in the comparative study, was that no difference in activity was observed between the normal nasal and infected sinus washings. Although it might be expected that the infected sinus material might have greater activity by virtue of their greater total mass it is apparent that the composition of the secretions in respect to the inactivation of the factors studied is not altered by infection.

The significance of the findings in relation to the possible establishment of infection is difficult to estimate. It is obvious that in reactions in which the third component of complement is essential, such as the properdin reaction (Pillemer et al, 1954), the presence of unphysiological concentrations of secretions might alter humoral resistance reactions. However, bacteria are removed from the upper respiratory pas-

sages primarily by mechanical means (Wilson and Miles, 1946). Therefore, the anticomplementary and anti-β-lysin activity of nasal secretions could be of importance only if the accumulation of mucus was present in amounts sufficient to prevent ciliary activity. Under these conditions complement-dependent and thermostable factors would be inhibited if they were present in the normal nasal secretions or in secretions produced by inflamed mucous membranes. It is also possible that respiratory tract mucus reaching the lower respiratory tract due to faulty ciliary action or to aspiration as may occur during anaesthesia may affect the humoral defense mechanisms of the lower respiratory tract and lead to the initiation of the pneumonic process.

SUMMARY

This investigation has dealt with the effect of human nasal secretions upon complement and β-lysin. Both normal nasal and infected sinus secretions were tested and found to inhibit these factors. Further experimentation demonstrated that the anticomplementary activity was due principally to the inactivation of the third component of complement. The fourth component was inactivated in some cases although higher concentrations of the secretions were necessary for this reaction. In addition, it was observed that the inhibition of β-lysin was due to a direct reaction between the secretions and the β-lysin. Alkaline shift in the pH of the serum when the tubes were closed only with a cotton plug was not responsible for the antimicrobial action of serum on the streptococcus.

Normal nasal and infected sinus secretions inactivated the third and fourth complement components of serum.

REFERENCES

Anderson, C. G., and Oag, R. K. 1939, Brit. J. Exper. Path. **20**: 25–32.

Ecker, E. E. and Gross, P. 1929, J. Infect. Dis. **44**: 250–253.

Ecker, E. E., Pillemer, L. and Seifter, S. 1943, J. Immunol. **47**: 181–193.

Eckstedt, R. D. 1954, A Study of the Antibacterial Activity of Normal Human Serum upon Selected Strains of Micrococcus Pyogenes, Doctoral Dissertation, University of Michigan, Ann Arbor, Michigan.

Engley, F. B. Jr. 1954a, Tex. Rep. Biol. & Med. **12**: 49–63.

———, 1954b. Tex. Rep. Biol. & Med. **12**: 236–249.

Fothergill, L. D., Dingle, J. H. and Chandler, C. A. 1937, J. Exper. Med. **65**: 721–734.

Fuller, A. T., Colebrook, L. and Mexted, W. B. 1939, J. Path. Bact. **48**: 443.

Gross, P. and Ecker, E. E. 1929, Proc. Soc. Exper. Biol. & Med. **26**: 508–510.

Hench, M. E. 1952, An Investigation of the Effect of Blood and Serum from Resistant and Susceptible Host Species on the Growth of Type 1 Pneumococcus, Doctoral Dissertation, University of Michigan, Ann Arbor, Mich.

Kabat, E. A. and Mayer, M. 1948, Experimental Immunochemistry, Springfield, Ill., Chas C Thomas Co.

Keefer, C. S. and Spink, W. W. 1938, J. Chem. Investigation, **17**: 23–30.

Lambert, H. P. and Richley, J. 1952, Brit. J. Exper. Path. **33**: 327–339.

Nungester, W. J., Wolf, A. A. and Jourdanais, L. F. 1932, Proc. Soc. Exper. Biol. & Med. **30**: 120–121.

Nungester, W. J., Jourdanais, L. F. and Wolf, A. A. 1936, J. Infect. Dis. **59**: 11–21.

Oerskov, J. 1940, Ztschr. Immunitätsforsch. **98**: 359–372.

Pillemer, L., Blum, L., Lepow I. H., Ross, O. H., Todd, E. W. and Wardlaw, A. C. 1954, Science, **120**: 279–285.

Pillemer, L. and Ecker, E. E. 1941, J. Biol. Chem. **137**: 139–142.

Smith, H. 1950, Biochem, J. **46**: 352–356.

Smith, H., Gallop, R. C., Harris-Smith, P. and Stanley, J. L. 1952, Biochem, J. **52**: 23–29.

THE EFFECT OF PENICILLIN ON MULTIPLYING AND NON-MULTIPLYING POPULATIONS OF SENSITIVE AND RESISTANT STRAINS OF FELINE PNEUMONITIS VIRUS

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The production of a penicillin-resistant strain of feline pneumonitis virus by repeated passage in the yolk sacs of chick embryos treated with penicillin was described in a previous report (Moulder, McCormack, Gogolak, Zebovitz, and Itatani, 1955). In the presence of sufficient penicillin to protect completely embryos inoculated with the parent virus, the resistant virus grew at one-half the normal rate and killed all infected embryos. In the range of 100 to 100,000 units per egg, the growth rate and lethality of the resistant strain were independent of the penicillin concentration.

The effect of penicillin on both non-multiplying and multiplying populations of parent and penicillin-resistant feline pneumonitis virus has now been studied in more detail. Treating non-multiplying suspensions of both viruses with very high concentrations of penicillin in vitro destroyed to an equal degree their ability to multiply upon subsequent inoculation into chick embryos. The action of penicillin on the two virus strains growing within the chick embryo yolk sac was almost entirely virustatic, and there was no evidence of a killing of multiplying virus analogous to the killing of growing bacteria by penicillin (Hobby, Meyer, and Chaffee, 1942; Hobby and Dawson, 1944; Chain and Duthie, 1945). In the presence of penicillin, the resistant virus appeared to be more capable of invading susceptible cells and initiating multiplication than was the parent virus.

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A portion of this work has been briefly reported elsewhere (Moulder, 1954).

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METHODS

Two strains of feline pneumonitis virus (Baker, 1944) were used. One was the stock virus maintained for many years by passage in the yolk sacs of chick embryos. Its general characteristics were described by Moulder and Weiss (1951). The other was a penicillin-resistant strain obtained by serial yolk sac passage in the presence of penicillin (Moulder et al, 1955). In the present work, the resistant virus was in its 48th and 49th penicillin passage.

Fertile White Leghorn eggs from a flock fed an antibiotic-free ration were incubated at 37 C for 6 days. The embryos were then inoculated via the yolk sac with 0.2 ml of a suitable dilution of infected yolk sac emulsion. In some experiments, penicillin and penicillinase were also inoculated by the same route, always in a volume of 0.2 ml. The embryos were reincubated and candled daily. Deaths prior to the 4th day after infection were regarded as nonspecific. When viruses of the psittacosis group are inoculated into the yolk sacs of chick embryos, there is a linear relationship between the logarithm of the inoculum size and the geometric mean of the survival time (average day of death) of the infected embryos (Golub, 1948; Crocker and Bennett, 1955). Table 1 demonstrates the existence of this relationship for feline pneumonitis virus and also shows that a constant virus concentration is reached at the

TABLE 1.—*Effect of inoculum size on average day of death and terminal virus concentration.*

Primary inoculation			Subinoculation	
LD_{50}'s inoculated	ADD*	Increase in ADD	Day yolk sacs harvested for subinoculation	ADD with 10^{-4} dilution of subinoculum
10^8	4.5		4	6.1
10^7	5.2	0.7	5	6.0
10^6	5.8	0.6	6	6.1
10^5	6.4	0.6	6	6.1
10^4	7.1	0.7	7	5.9

* Average day of death = geometric mean of the survival time.

Five groups of 10 6-day-old chick embryos were inoculated via the yolk sac with decimal dilutions of a crude yolk sac emulsion prepared from embryos infected with parent feline pneumonitis virus. At the indicated times, yolk sacs were harvested to prepare emulsions for subinoculation.

time of death, regardless of the size of the inoculum. By means of this relation, the virus content of an unknown sample may be compared with that of a standard preparation which has been titrated by a 50% endpoint method. In addition, identical aliquots of a single virus preparation may be subjected to different treatments either in vitro or in ovo and the resulting rates of virus multiplication may be compared in terms of the average day of death of groups of embryos receiving the different aliquots. For further description of chick embryo methods, see Moulder and Weiss (1951) and Moulder et al (1955).

Penicillin was determined by the filter paper disc method of Vincent and Vincent (1944). The Oxford strain of *Staphylococcus aureus* (ATCC no. 9144) was the assay organism. For each assay sample, the entire contents of 3 eggs were homogenized in a Waring blendor. When penicillinase was present, the homogenates were heated for 2 minutes at 100 C to inactivate the enzyme. This treatment did not decrease the pencillin content of control samples. Each sample was assayed in at least 2 dilutions on duplicate plates. For an unknown reason, when embryos were harvested and assayed immediately after penicillin administration, only 70 to 80% of the injected penicillin could be recovered. Therefore, assay values were expressed in terms of the percent original apparent concentration as determined by immediate assay.

Crystalline potassium penicillin G (benzyl penicillin) buffered with sodium citrate was dissolved in distilled water just before use. The penicillinase preparation used was Bacto-Penase Concentrate,* 1 ml of which is stated to destroy 500,000 units of penicillin in 2 hours at room temperature. All dilutions of virus, penicillin and penicillinase were made in veal infusion broth, pH 7.0.

* Difco Laboratories, Inc., Detroit, Michigan.

RESULTS

The effect of penicillin on nonmultiplying virus

While both multiplying and nonmultiplying bacteria take up penicillin from the medium and bind it so firmly that it cannot be dissociated by dilution or by treatment with a variety of agents (Maas and Johnson, 1949a and b; Rowley, Cooper, Roberts, and Smith, 1950), penicillin rapidly kills only growing bacteria and has no appreciable bacteriocidal effect on nongrowing cells (Hobby et al, 1942; Hobby and Dawson, 1944; Chain and Duthie, 1945). Because of this differential action of the antibiotic on multiplying and nonmultiplying bacteria, the action of penicillin on feline pneumonitis virus was investigated both in the test tube and in the chick embryo.

The first indication that a measurable interaction between virus and antibiotic occurs in vitro came from an abortive attempt to pass the resistant virus in the presence of penicillin by giving chick embryos a single inoculation of a mixture of penicillin and virus: the embryos so inoculated failed to die of the virus infection. Further study showed that the resistant virus may be almost completely inactivated by penicillin in vitro (fig. 1). When increasing amounts of penicillin were given to one series of embryos immediately after virus inoculation, the resistant virus exhibited a constant growth rate over the whole range of antibiotic concentration. A second series of embryos received exactly corresponding quantities of virus and penicillin, but instead of being injected separately, virus and drug were incubated together for 2 hours at 0 C before injection. The two diverging curves relating average day of death to penicillin concentration indicate that contact with penicillin concentrations of 100,000 units per ml and

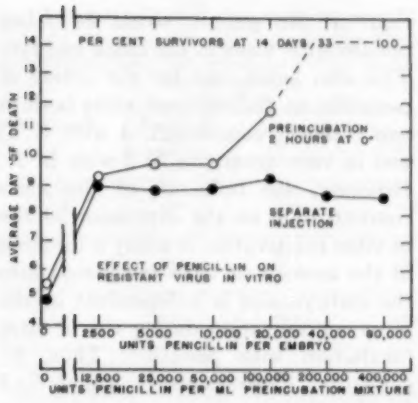

FIG. 1.—Effect of penicillin on penicillin-resistant feline pneumonitis virus in vitro and in ovo. See text for explanation. Reproduced from Moulder (1954) with the permission of *Bacteriological Reviews* and the Williams and Wilkins Company, Baltimore, Maryland.

greater progressively destroyed infectivity until complete inactivation occurred at 400,000 units. Blind subpassage of yolk sacs from embryos receiving the virus incubated with 400,000 units of penicillin per ml confirmed the absence of infective virus. These results are in accord with earlier studies on the inactivation of feline pneumonitis virus by penicillin in vitro. Hamre and Rake (1947) found that 100,000 units per ml caused a slight inactivation, while Eaton, Dozois, van Allen, Parish and Schawlm (1948) noticed no in vitro effect with 2000 units per ml.

Although nonmultiplying preparations of both parent and resistant virus are inactivated by contact with penicillin, most of the in vitro experiments were carried out with the resistant strain because its constant growth rate in embryos treated with a wide range of penicillin concentrations made it easier to differentiate between the action of penicillin in the incubation mixture and in the embryo receiving the preincubated virus and drug.

Specificity of the inactivation.—Since very high concentrations of penicillin are required to inactivate the virus, it is possible that this action of the antibiotic may be nonspecific and entirely unrelated to its antiviral action in ovo. While the following observations do not prove that the same properties of the penicillin molecule are responsible for both the in vitro inactivation and the in ovo inhibition of multiplication, they strongly suggest that the intact molecule is required for inactivation of the virus during in vitro incubation. First, when penicillin was inactivated by holding at pH 1 overnight at room temperature (Abraham and Chain, 1942), 400,000 units per ml had no effect on virus infectivity after 2 hours incubation at 0 C. Second, when virus inactivated by penicillin was incubated with penicillinase, a significant fraction of its original infectivity was restored (table 2). Eagle and Musselman (1949) have described somewhat analogous effects of penicillinase on penicillin-treated bacteria.

Factors affecting the degree of inactivation.—

1. Concentration of penicillin.—Penicillin-resistant feline pneumonitis virus was consistently inactivated by incubation with 200,000 to 400,000 units penicillin per ml, while 100,000 units

TABLE 2.—*Reversal by penicillinase of the in vitro inactivation by penicillin.*

Procedure	Effect on embryo survival	
	ADD	Percent survivors*
Inoculation of virus alone	5.5	0
Separate inoculation of virus and 800 units of penicillin	10.3	0
Preincubation of virus and penicillin		70
Preincubation of virus and penicillin followed by incubation with penicillinase	7.3	0

* Fourteen days after virus inoculation.

An undiluted yolk sac emulsion of penicillin-resistant virus was incubated with 400,000 units penicillin per ml for 2 hours at 0 C. The mixture was then diluted 1:10 and added to an equal volume of undiluted Bacto-Penase Concentrate. After 30 minutes at 37 C, this mixture was diluted 1:10 and 0.2 ml inoculated into 10 6-day-old embryos. Therefore, each embryo received the equivalent of 400,000/500 or 800 units of penicillin. Appropriate controls are described above.

TABLE 3.—*Effect of length of incubation on the in vitro inactivation by penicillin.*

Minutes incubation with penicillin	Units penicillin inoculated into each embryo	ADD*	Percent survivors†
0	0	5.5	0
0	800‡	10.3	0
5	800	10.0	10
30	800	12.0	33
120	800	12.9	60

* Survivors disregarded.
† Fourteen days after virus inoculation.
‡ Injected immediately after virus inoculation.
An undiluted yolk sac emulsion of penicillin-resistant feline pneumonitis virus was incubated with 400,000 units penicillin per ml for the indicated periods, and after 1:100 dilution, 0.2 ml of the penicillin-virus mixture was inoculated into groups of 10 6-day-old embryos.

occasionally produced some inactivation (fig. 1).

2. Length of incubation.—Table 3 indicates that the inactivating effect of 400,000 units penicillin per ml is negligible after incubation with the virus for 5 minutes at 0 C, is first apparent after 30 minutes, and continues to increase for as long as 2 hours.

3. Temperature of incubation.—The effect of temperature on the degree of inactivation was tested with an incubation mixture in which the concentration of penicillin, 100,000 units per ml, was such that there was a barely noticeable inactivation at 0 C. When the temperature was raised to 20 or 40 C, there was only a very slight increase in inactivation of the virus (table 4).

4. Concentration of virus.—Weiss (1950) showed that the smaller the inoculating dose, the more effective is penicillin in suppressing the multiplication of the parent strain of feline pneumonitis virus in the chick embryo. This also holds true for the action of penicillin on the resistant virus both in ovo (table 5, compare A. 3 with B. 3) and in vitro (compare A. 2 with B. 2). However, the influence of the virus concentration on the expression of the in vitro inactivation is solely a function of the amount of virus inoculated into the embryo and is independent of the virus concentration during the in vitro incubation with penicillin. Thus, by comparing experiments B. 2 and C. 2 in table 5, it is seen that when the same amount of virus is inoculated into chick embryos, a 10,000-fold difference in virus concentration during the in vitro incubation is without effect on the end result.

TABLE 4.—*Effect of temperature of incubation on the in vitro inactivation by penicillin.*

Temperature of incubation, C	ADD	
	No penicillin	Penicillin
0	5.8	6.6
20	5.8	6.8
40	5.9	7.1

An undiluted yolk sac emulsion of resistant feline pneumonitis virus was incubated at the indicated temperature for 2 hours with and without 100,000 units penicillin per ml. The mixture was diluted 1:10,000 and 0.2 ml inoculated into groups of 10 6-day-old chick embryos. Each embryo received 2 units penicillin in the inoculum; separate injection of 2 units of penicillin into embryos receiving the same amount of virus increased the ADD by 0.3.

These experiments may be interpreted as evidence for the in vitro formation of a firm, nondissociable bond between penicillin and feline pneumonitis virus, similar to that formed between penicillin and bacteria. When the concentration of penicillin is very high, the resulting penicillin-virus complex is incapable of reproduction when it is inoculated into chick embryos.

Relative sensitivity of parent and resistent virus to in vitro inactivation.—All the experiments so far described were carried out with the penicillin-resistant strain of virus. However, when the virus-penicillin incubation mixture is diluted 10,000-fold between incubation and injection into embryos, as in experiment C of table 5, the parent, penicillin-sensitive strain may also be used, because the concentration of penicillin in the diluted mixture is so small that it only slightly inhibits growth of the parent strain. Table 6 shows the results obtained in this kind of experiment with both parent and resistant virus. They demonstrate that the sensitivity to penicillin in vitro is

TABLE 5.—*Effect of virus concentration on the action of penicillin on penicillin-resistant virus in vitro and in ovo.*

Experiment	Virus concentration LD_{50} per ml		Penicillin concentration		Embryo survival	
	Incubated with penicillin in vitro	Inoculated into embryos	Incubated with virus in vitro, units per ml	Inoculated into embryos, units per embryo	ADD*	Embryo survivors†
A. 1	$10^{8.5}$	$10^{6.5}$	400,000	0	5.4	0
2		$10^{6.5}$		80,000	12.8	50
3		$10^{6.5}$		80,000	9.8	0
B. 1	$10^{5.5}$	$10^{3.5}$	400,000	0	6.6	0
2		$10^{3.5}$		80,000		100
3		$10^{3.5}$		80,000	12.5	77
C. 1	$10^{8.5}$	$10^{5.5}$	400,000	0	6.6	0
2		$10^{5.5}$		8		100
3		$10^{5.5}$		8	7.6	0

* Survivors disregarded.
† Fourteen days after virus inoculation.
Penicillin-resistant feline pneumonitis virus was incubated for 1 hour at 0 C with and without 400,000 units penicillin per ml. In experiment A, the crude virus emulsion was diluted 1:100 and 0.2 ml virus-penicillin mixture was injected into 10 six day embryos without further dilution. Experiment B differed from A only in that the virus dilution was 1:10,000. In experiment C, undiluted virus was incubated with penicillin and the mixture was diluted 1:10,000 before inoculation into chick embryos. The last line of data (3) for each experiment shows the effect on virus growth of an equal amount of penicillin inoculated into embryos immediately after virus.

almost exactly the same for both strains of virus. While equal sensitivity to inactivation does not necessarily mean equal uptake of penicillin, it is of interest that Eagle (1954) found that, in a number of bacteria, there was no correlation between development of resistance to penicillin and the ability to bind the antibiotic.

Effect of penicillin on multiplying virus

The effect of penicillin on feline pneumonitis virus multiplying within the cells of the chick embryo yolk sac was investigated by observing the effect on virus multiplication of the presence of penicillin in ovo for periods of 1 to 48 hours. Penicillin was removed after the desired time of contact by inoculating penicillinase into the yolk sac.

Figure 2 shows that, in the absence of penicillinase, about half of a 1000 unit dose of penicillin injected into the yolk sac disappeared from the whole egg in about 24 hours, while much smaller amounts were destroyed on succeeding

TABLE 6.—*Effect of penicillin concentration on the in vitro inactivation of parent and resistant virus.*

Virus	Penicillin concentration		Embryo survival			
			Inoculated with penicillin-virus incubation mixture		Separately inoculated with equivalent amounts of virus and penicillin	
	Units per ml in incubation mixture	Units inoculated per embryo	ADD*	Percent survivors†	ADD*	Percent survivors†
Parent	0	0	6.6	0		
	50,000	1	6.5	0		
	100,000	2	7.4	10	6.9	0
	200,000	4	8.9	20		
	400,000	8		100	8.9	0
Resistant	0	0	6.2	0		
	50,000	1	6.7	0		
	100,000	2	6.5	0	6.1	0
	200,000	4	8.0	20		
	400,000	8		100	7.7	0

* Average day of death. Survivors disregarded.
† Fourteen days after virus inoculation.
Undiluted yolk sac emulsion was incubated with penicillin for 2 hours at 0 C, diluted 1:10,000 and 0.2 ml inoculated into each of 10 6-day-old chick embryos.

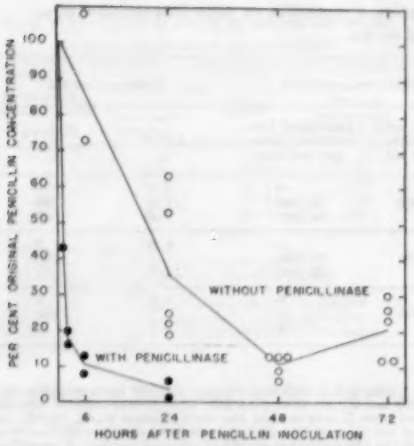

FIG. 2.—Disappearance of penicillin in chick embryos: effect of penicillinase on the rate of destruction. One thousand units of penicillin were injected into 6-day-old embryos via the yolk sac, and 0.04 ml Bacto-Penase Concentrate was injected via the same route immediately after the antibiotic. Each point represents the penicillin found in a pool of the entire contents of 3 eggs.

days. Such a biphasic curve of destruction has already been described by Hamre and Rake (1947). When 0.04 ml of the penicillinase preparation was injected at the same time, the penicillin was rapidly inactivated; only about 10% remained about 6 hours and practically none after 24.

Table 7 describes the effect on parent virus multiplication, as measured by embryo survival, of injecting penicillinase at different times after the simultaneous inoculation of virus and 1000 units of penicillin per embryo. One thousand units given at the time of virus inoculation completely protected the embryos for 10 days, so if penicillinase had not been injected, none of the penicillin-treated embryos would have died during the period of observation. The data show: (1) Penicillinase neither killed uninfected embryos nor altered the rate of virus multiplication in the absence of penicillin. (2) When virus, penicillin and penicillinase were simultaneously inoculated, the average day of death increased only slightly. Although the increase was small, it was constantly observed and probably represents the action of penicillin on the virus during the time required for its inactivation by penicillinase. (3) When penicillinase was inoculated into infected embryos at different times after penicillin injection, the inhibition of virus multiplication was proportional to the length of the delay between the penicillin and penicillinase injections. From these results, it was concluded that the administration of penicillinase in ovo provides a simple and specific method for controlling the time of contact between penicillin and virus within the chick embryo yolk sac.

TABLE 7.—*Effect of time of penicillinase injection on the effectiveness of penicillin in delaying the death of chick embryos infected with feline pneumonitis virus.*

Inoculum 10^{-3} LD_{50}'s virus and		Interval between penicillin and penicillinase injections	Embryo survival	
Penicillin 1000 units per embryo	Penicillinase*	Hours	ADD†	Percent survivors‡
—	+	No virus	—	100
—	—	—	4.2	0
—	+	—	4.2	0
+	—	—	—	100
+	+	0	4.6	0
+	+	6	5.1	0
+	+	12	5.9	0
+	+	24	6.0	0
+	+	36	6.5	0
+	+	48	7.2	25

* 0.2 ml of a 1:5 dilution of Bacto-Penase Concentrate.
† Survivors disregarded.
‡ Ten days after virus inoculation.
Groups of 10 6-day-old embryos were inoculated with parent virus and immediately thereafter with penicillin. Penicillinase was given at the times indicated above. All inoculations were via the yolk sac. Appropriate controls are shown in the first 4 lines of data. Administration of penicillinase alone at any time up to 48 hours after infection was without effect on the ADD.

Effect of simultaneous inoculation of penicillin and virus.—In the first series of experiments (fig. 3), 1000 units of penicillin was given each embryo only a few seconds after inoculation of either parent or penicillin-resistant virus, so that the antibiotic was present during

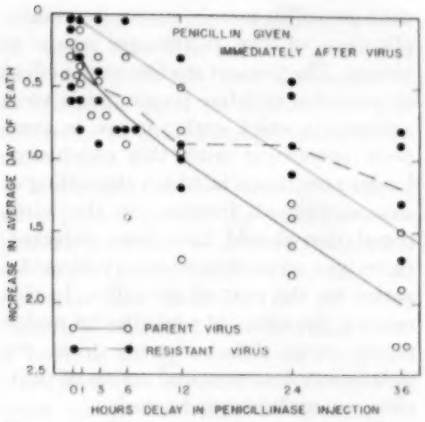

FIG. 3.—The effect of penicillin on parent and resistant virus multiplying in the chick embryo: penicillin administered immediately after virus inoculation. Four independent experiments were performed with each virus as described in table 7. The faint solid line represents the expected change in average day of death with time of contact with penicillin if the antibiotic is entirely virustatic.

adsorption of the elementary bodies on the host cells and their subsequent penetration and early development. Judging from the first appearance of initial bodies, active growth of the virus begins about 15 hours after inoculation (Weiss, 1949). Considering the results with both parent and resistant virus together, two conclusions may be drawn: (1) Penicillin almost immediately reacts with the virus, and its effect increases progressively with time of contact. (2) Although the presence of penicillin is noticeable at once, the effect of its removal is equally apparent. In other experiments, injection of penicillinase 72 hours after virus and penicillin still definitely increased embryo mortality. It appears that the constant presence of an inhibitory level of the antibiotic is necessary for continued protection of the embryo.

If the action of penicillin on multiplying virus is the same as its action on multiplying bacteria, then it should kill a large fraction of the virus growing in the cells of the yolk sac. However, if penicillin does not kill, but merely inhibits virus multiplication for the time it is present in the embryo, then a 12-hour exposure should lengthen the average day of death of infected embryos by 0.5, a 24-hour exposure by 1.0, etc. The light solid line in figure 3 describes such an expectation. The curve for parent virus falls below this line, but approaches it closely in slope. The most reasonable conclusion is that the action of penicillin is entirely virustatic and that the difference between expectation and observation is due to one or more of three factors: (1) Penicillin is not immediately destroyed upon injection of penicillinase (fig. 2). (2) The virus is subject to thermal inactivation during the postulated period of virustasis. (3) As in bacteria (Eagle and Musselman, 1949), the virus probably does not immediately begin to multiply as soon as the pencillin is destroyed but goes through a brief recovery phase. Each of these factors would tend to delay slightly the death of infected embryos.

Up to 12 hours exposure of the resistant virus to penicillin produces the the same effect as on parent virus (fig. 3). However, when resistant virus is in contact with penicillin for 12 to 36 hours in ovo, it is distinctly less sensitive to growth inhibition by the antibiotic and probably multiplies during this period. These experiments suggest that, in the presence of penicillin, the resistant strain is more capable of invading susceptible cells and initiating multiplication than is the parent virus.

Effect of inoculating penicillin 24 hours after infection.—In a second series of tests, penicillin was administered 24 hours after infection, when there was no extracellular virus and when rapid multiplication had begun (Weiss, 1949).

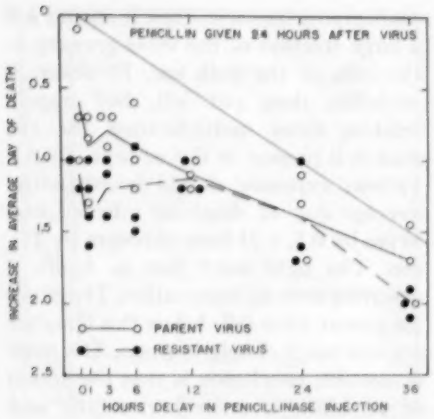

FIG. 4.—The effect of penicillin on parent and resistant virus multiplying in the chick embryo: penicillin administered 24 hours after virus inoculation. Three independent experiments were performed with each virus, and penicillin was given 24 hours after infection. Otherwise, conditions were as in figure 3.

Under these conditions, the immediate growth delaying action of the antibiotic was greater than when virus and penicillin were given together (compare fig. 4 with fig. 3). Since there was no extracellular virus present, penicillin must be able to penetrate the infected cell and react with intracellular virus. Under these conditions, there was also no evidence for a virucidal action of penicillin, and there was no difference in response between parent and resistant strains.

DISCUSSION

It has often been observed (see Quan, Meyer and Eddie, 1950; Hurst, Peters and Melvin, 1950) that, although acute infections with agents of the psittacosis group in mice and birds may be treated successfully with penicillin, the virus is not eradicated from the treated animals. They may relapse or virus may be recovered from their tissues long after therapy has ceased. For these reasons, it has been concluded that penicillin merely suppresses multiplication of the psittacosis group of viruses. The present studies on the effect of penicillin of feline pneumonitis virus growing in chick embryos are in complete agreement with this conclusion. Under conditions in which the killing of any significant fraction of the virus population should have been detected, there was no evidence for any virucidal action on the part of penicillin. In this respect, the effect of penicillin on multiplying virus differs sharply from the well-known bacteriocidal action of penicillin on multiplying bacteria.

The inability of penicillin to kill growing virus makes it difficult to explain the inactivation of feline pneumonitis virus by the drug in vitro. The irreversibility of the inactivation following great dilution of the virus-penicillin incubation mixture suggests that the virus firmly binds penicillin in a manner similar to bacteria, while the ineffectiveness of acid-inactivated penicillin and the restoration of infectivity by penicillinase point to the intact penicillin molecule as the inactivating agent. However, the necessity for a very high penicillin concentration in the in vitro incubation mixture and the lack of any virucidal action on the part of suppressive levels of the antibiotic in ovo indicate that the ability of penicillin to inactivate feline penumonitis virus in vitro and to suppress multiplication of the virus in ovo are probably unrelated properties of the penicillin molecule.

The behavior of parent and penicillin-resistant virus was so remarkably similar in these experiments that they offer little help in understanding the mechanism of acquired resistance to penicillin in feline pneumonitis virus. When penicillin was given at the same time the embryo was infected, the resistant strain multiplied more rapidly

than the parent after a period of about 12 hours. However, it is not certain if the magnitude of this difference is sufficient in itself to account for the difference in drug susceptibility of the two strains.

The ability of the virus to combine with penicillin and the many resemblances between the action of penicillin on feline pneumonitis virus and on bacteria (see Moulder et al, 1955) furnish a mass of evidence which in its entirety is strong support for the belief that penicillin directly interferes with some unknown metabolic process in the virus which is vital to its reproduction. However, the failure to find any killing of actively growing virus by penicillin is not in agreement with the bacterial studies and offers the caution that the analogy with the bacterial mode of action should not be pushed too far.

SUMMARY

When a stock strain of feline pneumonitis virus and a penicillin-resistant strain derived from it were incubated in vitro with penicillin G for 2 hours at 0 C, the viruses lost almost all their infectivity for chick embryos when the penicillin concentration was 200,000 to 400,000 units per ml.

The degree of in vitro inactivation was dependent on penicillin concentration and time of incubation. It was virtually independent of temperature and virus concentration. Ten thousand-fold dilution of the virus-penicillin incubation mixture did not restore infectivity. Acid-inactivated-penicillin was without effect, and treatment with penicillinase partially reversed the inactivation.

Parent and penicillin-resistant virus were equally susceptible to in vitro inactivation by penicillin.

It was concluded that virus and penicillin form a nondissociable bond in vitro and that when the penicillin concentration is very high, the resulting virus-penicillin complex cannot reproduce when inoculated into chick embryos.

The effect of penicillin on the two strains of virus growing in the chick embryo yolk sac was investigated by observing the effect on virus multiplication of the presence of penicillin in ovo for periods of 1 to 48 hours. Penicillin was removed after the desired time of contact by inoculating penicillinase into the yolk sac.

As judged by its subsequent effect on virus multiplication in the embryo, penicillin immediately reacted with the virus and growth inhibition was proportional to time of contact. The action of penicillin on multiplying virus appeared to be entirely virustatic and not virucidal; this is in contrast to its action on multiplying bacteria.

In the presence of penicillin, the resistant virus was more capable of invading susceptible cells and initiating multiplication than was the parent virus.

REFERENCES

Abraham, E. P. and Chain, E. 1942, Brit. J. Exper. Path. **23:** 103–115.

Baker, J. A. 1944, J. Exper. Med. **79:** 159–172.

Chain, E. and Duthie, E. S. 1945, Lancet, **268:** 652–657.

Crocker, T. T. and Bennett, B. M. 1955, J. Immunol. **75:** 239–248.

Eagle, H. 1954, J. Exper. Med. **100:** 103–115.

Eagle, H. and Musselman, A. D. 1949, J. Bact. **58:** 475–490.

Eaton, M. D., Dozois, T. F., van Allen, A., Parish, V. L. and Schawlm, S. 1948, J. Immunol. **58:** 251–267.

Hamre, D. and Rake, G. 1947, J. Infect. Dis. **81:** 175–190.

Hobby, G. L. and Dawson, M. H. 1944, Proc. Soc. Exper. Biol. & Med. **56:** 181–184.

Hobby, G. L., Meyer, K. and Chaffee, B. 1942, Proc. Soc. Exper. Biol. & Med. **50:** 281–285.

Hurst, E. W., Peters, J. M. and Melvin, P. 1950, Brit. J. Pharmacol. **5:** 611–624.

Golub, O. J. 1948, J. Lab. & Clin. Med. **33**: 1241–1248.

Maas, E. A. and Johnson, M. J. 1949a, J. Bact. **57**: 415–422.

———, 1949b, J. Bact. **58**: 361–366.

Moulder, J. W. 1954, Bact. Rev. **18**: 170–176.

Moulder, J. W. and Weiss, E. 1951, J. Infect. Dis. **88**: 56–67.

Moulder, J. W., McCormack, B. R. S., Gogolak, F. M., Zebovitz, M. M. and Itatani, M. K. 1955, J. Infect. Dis. **96**: 57–74.

Quan, S. F., Meyer, K. F. and Eddie, B. 1950, J. Infect. Dis. **86**: 132–135.

Rowley, D., Cooper, P. D., Roberts, P. W. and Smith, E. L. 1950, Biochem. J. **46**: 157–163.

Vincent, J. G. and Vincent, H. W. 1944, Proc. Soc. Exper. Biol. & Med. **55**: 162–164.

Weiss, E. 1949, J. Infect. Dis. **84**: 125–149.

———. 1950, J. Infect. Dis. **87**: 249–263.

THE EFFECT OF THYROXIN ON RESPIRATION AND VIRUS GROWTH IN CHICK EMBRYO TISSUE CULTURES

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Inhibition of the growth of influenza virus accompanied by an initial increase in oxygen consumption has been demonstrated in tissue cultures and deembryonated eggs with substances which are known to uncouple phosphorylation from oxidation, such as 2,4-dinitrophenol[1] and alkyl esters of 3,5-di-iodo-4-hydroxybenzoic acid.[2] Previous reports of preliminary experiments[2] indicated that relatively high concentrations of dl-thyroxin were slightly inhibitory to growth of influenza virus and also to proliferation of chorioallantoic epithelium, and oxygen consumption. Observations by Weiss, Moulder and Itatani[3] had shown that thyroxin increased the susceptibility of mice to infection with murine pneumonitis virus although no increased titer of virus in the lungs of thyroxin-treated animals was found.

Thyroxin has been reported to have an uncoupling effect in mitochondrial preparations both from mammalian cells[4,5] and from mycobacteria[6] and possibly on intact bacteria.[7] Recent observations in our laboratory indicate that the action on influenza virus in tissue culture of l-thyroxin and l-tri-iodothyronine in low concentrations is quite different from that of uncoupling agents such as 2,4-dinitrophenol in that normal or increased growth of virus accompanies an increase in the oxygen consumption of the tissue. There is also evidence of an increased rate of tissue damage in infected preparations containing thyroxin which would seem to be in accord with the observations of Weiss et al on pneumonia in mice.

Several factors affecting the action of thyroxin on growth of influenza virus have been investigated in an attempt to uncover the reasons for the irregular stimulation by this substance. Perhaps the most interesting result of these investigations has been the discovery that adenosine phosphates change the effect of thyroxin on virus growth from one of slight stimulation to partial inhibition without reduction in the rate of oxygen consumption.

MATERIALS AND METHODS

Virus.—The PR8 allantoic passage strain of influenza A was titrated in tissue cultures and the dilution of the inoculum used in experiments was adjusted to approximately 100 tissue culture infective doses. The actual amount of inoculum was 0.1 ml of a dilution of allantoic fluid between 10^{-3} and $10^{-4.5}$. This produced the first detectable hemagglutinins in the tissue culture at 16 to 20 hours. Growth of virus was measured by hemagglutination and by infectivity titrations in the allantoic sac of intact chick embryos (EID_{50}).

Thyroxin and tri-iodothyronine were used as the

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1. Eaton, M. D. and Perry, M. E. 1953, J. Infect. Dis. **93**: 269–277.
2. Eaton, M. D., Adler, L. T. and Perry, M. E. 1953, Proc. Soc. Exper. Biol. & Med. **84**: 57–60.
3. Weiss, E., Moulder, J. W. and Itatani, M. K. 1952, J. Infect. Dis. **90**: 21–29.
4. Hoch, F. L. and Lipmann, F. 1953, Fed. Proc. **12**: 218.
5. Maley, G. F. and Lardy, H. A. 1953, J. Biol. Chem. **20**: 435–444.
6. Brodie, A. F. and Gray, C. T. 1956. J. Biol. Chem. **219**: 853–862.
7. Wainfan, E. and Marx, W. 1955, J. Biol. Chem. **214**: 441–445.

synthetic 1-forms, which were obtained through the courtesy of Dr. A. E. Hemming of Smith, Kline and French. A few experiments were done with preparations of dl-thyroxin sodium bought from the Delta Chemical Company and Nutritional Biochemicals, Incorporated. The thyroxin or tri-iodothyronine was suspended in phosphate buffer and sufficient NaOH added to dissolve the substance. The resulting solution was sterilized by filtration.

Tissue culture methods.—The preparation of tissue cultures from minced 10- to 12-day chorioallantoic membrane has been described previously. Two types of culture were used: shaken cultures in 50 ml Erlenmeyer flasks[1,8] and roller tube cultures.[8] Each tube or flask contained 0.05 ml of tissue in 4 ml of Hanks' balanced salt solution (BSS). Since the experiments were of short duration, 1 to 3 days, migration and proliferation of tissue in the roller tubes was obtained without the addition of serum or plasma. The temperature of incubation was 37 C.

Substrates.—In previous studies[1,2,8] it has been demonstrated that sodium pyruvate* permits growth of virus and maintenance of pH without $NaHCO_3$ in tissue cultures loosely covered to permit escape of CO_2. Combinations of pyruvate and glucose, 0.1% and 0.02%, respectively, permit somewhat better growth of virus with little drop in pH. These substrates were used both in shaken flasks and roller tubes. When glucose 0.1% was used by itself, addition of the usual amount of $NaHCO_3$ and tight closing of the roller tubes with rubber stoppers was essential. With glucose in the medium the drop in pH was somewhat more rapid in the presence of thyroxin than in the controls.

Measurements of oxygen consumption were done by the conventional method of Warburg. After incubation for 24 to 72 hours the tissue fragments from cultures in roller tubes or flasks were collected by centrifugation and washed twice with BSS. The volume of tissue (approximately equivalent to wet weight) was measured in a protein-determination centrifuge tube after packing at 1500 r.p.m. for 15 minutes in a standard International Centrifuge. The tissue was suspended in 2 cc of balanced salt solution containing 0.1% glucose or pyruvate and the rate of oxygen consumption measured over a period of 3 hours.

Tissue viability.—In addition to the measurements of oxygen consumption, survival of tissue was evaluated by direct microscopic examination of fibroblasts and epithelial sheets sticking to the glass wall of the roller tubes and by measurements of the volume of tissue remaining after incubation. Since influenza virus seems to grow mainly in the epithelial components of the culture and not in fibroblasts,[9] most significance was attached to the growth or extension of the epithelial cells.

RESULTS

Effect of thyroxin on oxidation of pyruvate and glucose by chorioallantoic tissue.—Minced, washed membrane was incubated in shaker flasks for 24, 48 or 72 hours with BSS containing 0.1% pyruvate, 0.02% glucose and 10 μg/ml of thyroxin. Control tissue was similarly incubated without thyroxin. Measurements of respiration were then done in samples of treated and control tissue setting up 3 sets of Warburg flasks with glucose, pyruvate, and no substrate, respectively. No thyroxin was added to the Warburg vessels. The results are presented in table 1.

At the end of 24 hours the rate of oxygen consumption of tissue which had been incubated with thyroxin was approximately twice as great as that of the controls and most of the respiration was endogenous (not increased by presence of substrate). When tissue was incubated for 48 hours a stimulatory effect of glucose or pyruvate on oxygen consumption was observable both in thyroxin-treated and normal tissue and the increased rate resulting from thyroxin was again evident. By 72 hours the samples of tissue incubated with thyroxin showed no significant difference from the controls tested with the same substrate. Although stimulation of respiration in the Warburg was produced in both series by pyruvate, glucose produced little increase of oxygen consumption of the control tissue and none in that pre-incubated for 72 hours with

8. Daniels, J. B., Eaton, M. D. and Perry, M. E. 1952 J. Immunol. **69**: 321–329.

* Nutritional Biochemicals reagent grade.

9. Stulberg, C. S. and Shapira, R. 1953, J. Immunol. **70**: 51–59.

TABLE 1.—*Substrate effects on tissue respiration in Warburg.*

Thyroxin μg/ml*	Warburg substrate	Time of pre-incubation*					
		24 hours		48 hours		72 hours	
		Q O_2†	Ratio‡	Q O_2	Ratio	Q O_2	Ratio
10	0.1% glucose	540	1.9	420	2.5	142	0.87
10	0.1% pyruvate	640	2.3	520	3.0	310	1.9
10	None	570	2.0	306	1.8	176	1.1
0	0.1% glucose	320	1.1	300	1.8	227	1.4
0	0.1% pyruvate	320	1.1	370	2.2	365	2.2
0	None (control)	280	(1.0)	170	(1.0)	165	(1.0)

* Tissue pre-incubated with or without thyroxin in BSS with 0.1% pyruvate and 0.02% glucose in shaker flasks for periods indicated before Warburg measurements. The latter were done for a period of 3 hours on washed tissue without added thyroxin.
† Q O_2 = μl per gram (wt weight) of tissue per hour.
‡ Ratio of Q O_2 indicated to Q O_2 of control (last line of table).

thyroxin. This late depressive effect of thyroxin on respiration has been repeatedly observed in other experiments.

A latent period before the appearance of the stimulatory effect of thyroxin on respiration was indicated by experiments in which the oxygen consumption was followed during the first 18 hours. This was done either by direct measurements of respiration of tissue in Warburg flasks with and without thyroxin, or by preincubation of tissue in roller tubes for periods from 3 to 18 hours followed by short-term measurements of oxygen consumption.

It has been found that the rate of oxygen consumption of normal tissue incubated in the Warburg flasks for periods of 24 hours falls more rapidly than that of tissue maintained in flasks on a slower rocking mechanism or in roller tubes. Unfavorable conditions for tissue survival in the Warburg apparatus, either as a result of the rapid shaking or of removal of CO_2, is indicated by these observations. Even in the shaken flask or roller tubes, there was a gradual decrease in oxygen consumption of the control tissue. Consequently, an absolute increase of respiration with thyroxin was seldom observed, but it was found that the ratio of QO_2 thyroxin: QO_2 control did increase after 6 to 12 hours of incubation. From the results of a representative series of experiments shown in table 2 it may be seen that a relatively high concentration (10 μg/ml) of thyroxin was required for demonstration of the increase in respiration when the entire experiment was done in the Warburg apparatus, while as little as 0.1 μg/ml was stimulatory when preincubated with the tissue in roller tubes for 6 to 12 hours.

Effect of infection and thyroxin on growth of epithelium and oxygen consumption.—In general, the effect of thyroxin on tissue metabolism was biphasic; stimulation during the 1st day and depression after the 2nd or 3rd day. As the

TABLE 2.—*Time of appearance of stimulatory effect of thyroxin on tissue respiration.*

Preincubation	Thyroxin μg/ml	Q O_2—thyroxin over control at hour:							
		3		6		12		18	
			R		R		R		R
No*	10	355/350	1.02‡	317/316	1.0	373/308	1.21	305/230	1.33
No	1	344/350	0.98	291/316	0.92	298/308	0.97	215/256	0.91
Yes†	1					405/283	1.43	316/301	1.05
Yes	0.1			360/307	1.17	304/230	1.32	470/330	1.42

* Direct Warburg measurement on fresh tissue with 0.1% glucose. 0.1% $CaCO_3$ was added to Warburg flasks to maintain pH at 7.6.
† Pre-incubation in roller tubes for 6, 12, or 18 hours with 0.1% glucose in BSS followed by measurement of O_2 in Warburg flasks with 0.1% glucose (no $CaCO_3$).
‡ Figures under R represent ratio of oxygen consumption in thyroxin group to controls.

data in table 1 indicate the late depression is somewhat more obvious when the respiration of tissues that have been incubated with thyroxin is measured in the presence of 0.1% glucose than in measurements with pyruvate. Since all Warburg measurements except those referred to in table 1 were done with 0.1% glucose, the results presumably reflect the late depression by thyroxin to a maximum extent.

Although certain influenza virus strains (including PR8) produce some degeneration of chorioallantoic epithelium[10] and embryonic lung tissue,[11] this cytopathogenic effect is not as well defined as it is with other viruses and tissue culture systems. Microscopically visible signs of epithelial degeneration reach a maximum at 4 to 5 days after inoculation of virus.[10] In the present experiments the distance of extension of the epithelial sheet from the original central fragment of tissue was graded + to +++, and the effects during the first 3 days were taken to represent the balance between factors leading to degeneration and the tendency of the epithelial sheet to extend. From the data in table 3 it may be seen that growth of epithelium in the presence or absence of virus was about the same after the 1st day of incubation but on the 2nd day there was somewhat less extension of epithelium in the tubes containing virus. Thyroxin in the amounts of 0.1 to 1.0 μg/ml generally caused depression of epithelial growth either in the presence or absence of virus.

Measurements of oxygen consumption showed that in the presence of thyroxin and virus infection the ratio O_2 thyroxin: O_2 controls at 2 days was slightly but consistently less than the corresponding ratio for uninfected tissue cultures. In agreement with the results of other investigators,[12] we have been unable to find a difference between the rate of oxygen consumption in infected and normal tissues in the absence of thyroxin. It may be, therefore, that the thyroxin speeds up the cytopathogenic effect of the virus, as well as increasing its rate of growth, and this effect becomes measurable as a depression of oxygen consumption by the 2nd day.

Autolysis of tissue.—In roller tube or flask cultures under the conditions of these experiments the total amount of

10. Daniels, J. B. and Eaton, M. D. Unpublished observations.
11. Ledinko, N. 1955, Nature, **175**: 999–1001.
12. Ackerman, W. W. 1951, J. Biol. Chem. **189**: 421–427.

TABLE 3.—*Tissue growth and oxygen consumption after preliminary incubation in roller tubes.*

Medium*	Thyroxin μg/ml	Virus	Epithelium T/C† day		O_2 ratio T/C† at day	
			1	2	1	2
PG	1.0	+	++/++	+/++	1.15 (4)‡	0.94 (6)
PG	1.0	0	++/++	+/+++	1.45 (5)	1.20 (7)
G	1.0	+	++/++	±/++	1.48 (6)	0.69 (5)
G	1.0	0	++/++	+/+++	1.24 (2)	0.83 (2)
G	0.1	+	—	—	1.35 (3)	0.95 (3)
G	0.1	0	—	—	1.12 (1)	1.13 (2)
O	1.0	+	++/++	+/+	0.95 (1)	0.65 (1)
O	1.0	0	++/++	+/++	1.50 (1)	0.78 (1)
O	0.1	+	++/++	±/+	1.10 (2)	0.49 (1)
O	0.1	0	++/++	++/++	1.24 (2)	1.07 (1)

* Roller tube cultures contained the following mediums:
PG = BSS containing 0.1% pyruvate and 0.02% glucose.
G = BSS containing 0.1% glucose.
O = BSS with no substrate.
† T/C values for tubes with thyroxin over corresponding value for control. For oxygen consumption ratios of the rates in cu mm/g/hr of tissues previously incubated with thyroxin to that of control.
‡ Figures in parentheses represent number of experiments averaged in result given

TABLE 4.—*Effect of thyroxin and virus on autolysis of tissue.*

Thyroxin μg/ml	Virus	Ml of tissue recovered on day*			
		1	2	3	3S†
1.0	+	0.16 (5)	0.12 (11)	0.08 (3)	0.09 (10)
1.0	0	0.15 (5)	0.11 (11)		0.13 (4)
10.0	+			0.07 (5)	
0	+	0.17 (5)	0.13 (11)	0.11 (8)	0.15 (10)
0	0	0.18 (5)	0.14 (11)		0.17 (4)

* Volume of tissue recovered from 4 flasks or roller tubes. Total volume of tissue added at beginning of experiment was 0.05 ml per culture or 0.20 ml total.
† Column 3S gives results with shaken flasks at 3 days; other data are for roller tube cultures. Figures in parentheses give number of experiments averaged.

tissue recoverable by collecting it and centrifuging in a graduated tube does not increase, but rather tends to decrease over a period of 3 days, due to autolysis of the original fragments. Assuming some loss from technical difficulties, about 15 to 25% of the tissue, as shown in the last line of table 4, disappears over a period of 2 to 3 days. In the presence of 1 μg/ml thyroxin by itself the amount of autolysis appears to be slightly greater than in the controls, but this is not as much as in cultures containing both virus and thyroxin where 50 to 60% of the tissue has disappeared by 3 days. Increased metabolism from thyroxin combined with destruction of tissue by virus appear, therefore, to be reflected in these quantitative measurements of total volume of tissue, and the data support the microscopic observations and the measurements of oxygen consumption.

Effect of thyroxin on growth of virus.—We must first of all make it clear that the stimulatory effects of thyroxin on growth of virus were much more variable than the action on oxygen consumption and survival of tissue described in the previous sections. Much work was done in an effort to account for the variations, and although some of the factors concerned appear to have been recognized there may be others such as individual, seasonal, or nutritional variations in the chick embryos which are difficult or impossible to control.

The results of three representative experiments in which infectivity and hemagglutinins were titrated in parallel are presented in table 5. With the preparation of l-thyroxin 2- to 20-fold stimulation of viral growth was often observed at the end of 18 to 24 hours, but by 48 hours the values for thyroxin-treated and control cultures were similar. The infectivity/HA ratios (log infectivity minus log HA) were calculated for the individual titrations in each of these experiments and the range of these ratios is set forth in the last column. It is evident that the ratios

TABLE 5.—*Effect of l-thyroxin on infectivity and hemagglutinin titers.*

Exp. no.	l-thyroxin μg/ml	Medium	18 hours		24 hours		48 hours		Inf:HA† range (log)
			Log EID_{50}*	HA	Log EID_{50}	HA	Log EID_{50}	HA	
1	1.0	G			6.8	19	7.3	105	5.3–5.5
	0	G			5.7	1	7.3	70	5.5–5.7
2	1.0	G	6.5	9	7.3	38			5.5–5.7
	0	G	6.3	7	6.9	32			5.4–5.5
3	0.1	O	6.3	3	7.5	40		112	5.8–5.9
		O	5.5	<2	6.9	9		88	5.9

* EID_{50} = log 50% egg infectious doses per 0.1 ml.
HA = reciprocal of hemagglutination titer (each number is average of 3 or 4 individual tissue cultures)
† Log EID_{50} minus log HA titer.

give no indication that thyroxin causes either the formation of "incomplete" virus or of unusually large amounts of infectious virus.

In a long series of experiments the effects of l-thyroxin at concentrations of 0.01 to 50 μg/ml and of l-tri-iodothyronine at 0.001 to 1.0 μg/ml were studied. It soon became evident that the stimulation of virus growth was not directly related to the concentration of these substances in the tissue culture medium. In fact, under certain conditions the higher concentrations tended to be slightly inhibitory.

In table 6 the results of infectivity and hemagglutinin titrations and of measurements of oxygen consumption are presented as T/C ratios. The averaged values for the actual EID_{50} and hemagglutination titrations of the control cultures in each group are given in table 6a. It will be noted that the amount of virus in the inoculum was adjusted so that measurable hemagglutinins first appeared at about 18 hours and reached a maximum at 48 hours. Growth of virus was slower in the absence of substrate, or in those experiments where pyruvate or glutamate but no glucose was present, than in cultures containing glucose. In the experiments using 0.1% pyruvate or glutamate alone or in combination with 0.02% glucose, the T/C ratios for infectivity at 18 hours and hemagglutinins at 24 hours gave evidence of slight stimulation of virus growth by thyroxin at levels of 0.01 to 1.0 μg/ml and tri-iodothyronine at 0.1 to 0.01 μg/ml. In the presence of glucose alone thyroxin at 1.0 μg/ml or tri-iodithyronine at 0.01 μg/ml tended to be slightly inhibitory after the first 24 hours of incubation, but with 10-fold lower concentrations of these substances there was a slight stimulatory effect. When the medium contained no substrate the stimulatory effects of thyroxin on virus growth were still observable and, in fact, appeared more definite than in the presence of glucose (last line of table 6).

The results for virus growth and oxygen consumption presented in table 6 disclose a biphasic pattern of thyroxin and tri-iodothyronine, that is, early stimulation when the ratios rise above 1 followed by depression when the ratios become 1 or less. The late depressive effect on oxygen consumption can be minimized by lowering the concentration to 0.01 μg/ml in the case of thyroxin or 0.001 μg/ml of tri-iodothyronine.

If addition of virus were done at 24 hours after the thyroxin, it might be expected that some inhibition would

TABLE 6.—*Effect of substrate and amount of thyroxin or tri-iodothyronine on virus growth.*

l-thyroxin μg/ml	l-tri-iodo thyronine μg/ml	Substrate	Ratio T/C at hour*:				O_2—T/C at hour:		
			18 EID_{50}	24 HA	48 HA	72 HA	18	48	72
1.0		G	8	0.9	0.9		1.5	0.70	
0.1		G	13	1.8	1.0		1.4	0.95	
	0.01	G		0.8	0.7		1.2	1.1	
	0.001	G		1.7	1.4		1.2	1.1	
1.0		PG	5		1.4	1.4	1.2	0.95	0.50
0.1		PG	7		1.5	1.1	1.6	0.75	0.41
0.01		PG	7		1.8	1.1	2.1	1.4	0.77
	0.1	PG		2.1	0.9		1.2	0.70	
	0.01	PG		2.7	1.1		1.2	1.0	
0.1		P†		7.0	4.3	4.8			1.7
0.1		GlG†		3.0	1.3		1.4	0.95	
0.01		GlG†		1.1	1.1				0.95
0.1		Gl†		3.0	2.5	2.4			0.35
0.1		O	7	4.5	1.4		1.1	0.49	

* All ratios in this table are arithmetical. Two to six experiments averaged.
† P =0.1% pyruvate, GlG =0.1% glutamate and 0.02% glucose, Gl =0.1% glutamate.
Other substrates as in table 3.

TABLE 6a.—*Infectivity and hemagglutination titers of controls for table 6.*

Substrate	Titers at hour:				
	18 EID_{50}	18 HA	24 HA	48 HA	72 HA
G	$10^{6.1}$	8	61	120	
PG	$10^{6.4}$	8	60	110	50
P			5	14	19
GlG		10	80	210	150
Gl			17	45	51
O	$10^{6.7}$	<2	4	36	

occur, because by this time the action of the drug might have reached the depressive phase. Experiments were done to test this hypothesis, but the results were so irregular that no definite conclusions could be drawn. The main difficulty probably was attributable to the lack of a method for exact timing of the biphasic effects of the thyroxin.

Effect of large doses of l- and dl-thyroxin.—It has been reported that the uncoupling effect with mitochondria (lower P/O_2 ratios) is produced equally by d- and l-thyroxin.[6] If this effect were produced by high concentration on intact cells in tissue culture, then inhibition of virus growth might be expected with both l- and dl-thyroxin. To test this possibility experiments were carried out with two commercial preparations of racemic compound and the results were compared with those obtained with large doses of the l-thyroxin which had been used in the experiments already described. With both of the dl-preparations inhibition of virus growth was frequently obtained at concentrations of 10 to 50 μg/ml, while no inhibition was seen with the l-thyroxin at this concentration (table 7). In a few experiments, however, stimulation was observed with the Delta and NB preparations as well as with the SKF. Stimulation of oxygen consumption at 24 hours appeared to be quite uniform with all preparations. Inhibition of the extension and proliferation of epithelium was not noticeably greater with the dl-preparations than with the l-thyroxin.

TABLE 7.—*Variable effects of preparations of dl-thyroxin on virus growth.*

Thyroxin	μg/ml	Number of experiments	Ratio HA T/C		O_2-T/C 24 hours
			Range	Average	
Delta (dl-)	50	14	0.19–2.5	0.63	1.5
	10	16	0.53–2.5	0.85	1.36
NB (dl-)	50	3	0.25–2.1	1.0	1.3
	10	3	0.4 –1.6	0.93	1.4
SKF (l-)	50	4	1.0 –2.7	1.7	1.37
	10	6	1.0 –2.3	1.5	1.95

A possible limiting factor in these experiments is the solubility and diffusibility of thyroxin. Concentrations of 50 μg/ml presumably would tend to come out of solution at a pH near neutrality. A tendency of thyroxin to increase acid formation from glucose in tissue cultures has been observed and it is possible, therefore, that in many of the experiments the full concentration was present only during the first 12 to 24 hours before the pH fell below 7.6. In one series of experiments with dl-thyroxin at 10 and 50 μg/ml the effect of initial pH levels of 7.0, 7.4, 7.8 and 8.2 was examined, and an attempt was made to maintain the pH at the specified level by periodic addition of bicarbonate. The results failed to demonstrate any relation of pH to stimulation or inhibiton of virus growth or oxygen consumption by dl-thyroxin.

The data in table 7 suggest that l-thyroxin by itself even in large doses does not produce significant inhibition. When both d and l isomers are present together slight to moderate inhibition of virus growth is usually observed.

Action of adenosine phosphates.—The irregular effects of thyroxin might be the result of some biochemical variation in the chick embryo tissue. In studies on energy transfer the activity of adenosine-triphosphatase often must be controlled, but the use of NaF for this purpose was not feasible in our

experiments because of its many secondary toxic effects. Attempts were made, therefore, to counteract possible deficiencies of adenosine triphosphate (ATP) within the cells by supplying an excess of this compound in the external medium.

Addition of ATP at concentrations of 0.5 to 1.0 mg/ml was found to be quite inhibitory by itself to the growth of virus. Since at this level a precipitate formed in the medium, removal of Ca^{++} by ATP or by free phosphate seemed a likely reason for the observed inhibition of virus.[13] At concentrations of 0.2 mg/ml no precipitation was evident and only slight inhibition of virus growth was found. Control experiments were done with sodium phosphate added at about 1 to 3 times the concentration of $PO_4^{\equiv}$ which would be furnished by the phosphate residue of the ATP. At this concentration of inorganic phosphate there was no inhibition of virus even though higher levels are known to inhibit.[13]

In the presence of thyroxin, ATP was found to exert a significant inhibitory effect on hemagglutinin production at 18 hours, that is, at a time when the thyroxin alone was stimulatory. This inhibitory effect was, however, only temporary. By 48 hours the T/C ratio of the HA titers has risen to a value approaching 1.0 in those cultures containing both ATP and thyroxin. The results of these experiments are presented in table 8. With adenosine monophosphate (muscle adenylic acid) similar effects were observed.

Adenosine when added at 0.1 mg/ml, although about as inhibitory to virus growth at ATP at 0.2 mg/ml, did not reverse the stimulatory action of thyroxin. Phosphocreatine* at concentrations of 1.0 and 0.2 mg/ml was inert with or without thyroxin. Also disodium phosphate added at concentrations up to 0.5 mg/ml had no apparent reversing effect on thyroxin.

TABLE 8.—*Effect of adenosine phosphates in reversing growth stimulation by thyroxin.*

Thyroxin μg/ml	T/C ratios of HA titers at 18 hours with:				
	ATP* 0.2 mg/ml	AMP* 0.2 mg/ml	AMP 1.0 mg/ml	Adenosine 0.1 mg/ml	Na_2HPO_4 0.5 mg/ml
1.0	0.33	0.56	0.25		
0.1	0.19	0.27	0.10	12.0	5.0
0	0.50	1.0	0.48	0.40	1.0

* ATP = adenosine triphosphate.
AMP = adenosine-5-phosphate.

ATP and AMP had no demonstrable effect under the conditions of these experiments on the early stimulation of oxygen consumption by thyroxin or the secondary depression in tissue respiration observed after 48 to 72 hours. The growth and extension of epithelium was not visibly affected by ATP, AMP or adenosine at concentrations of 0.1 to 0.2 mg/ml, and since the initial inhibitory effects on virus growth of these substances with thyroxin tended to be reversed spontaneously their action cannot be attributed to nonspecific tissue damage.

DISCUSSION

The effects of thyroxin on virus growth, although small in magnitude, show a tendency opposite from that observed with other agents which uncouple phosphorylation from oxidation. A biphasic effect of thyroxin on growth and differentiation has been repeatedly described with different tissue systems; for example, in stimulating metamorphosis of tadpoles but inhibiting growth,[14] increasing growth of fibroblasts at 1 μg/ml but with late inhibition at higher concentrations,[15] and more

13. Gohd, R. S. and Huang, J. 1955. Bact. Proc. (Meeting of May 8–12), pp. 62–63.

* Sigma Chemical Company.

14. Zondek, H. 1936, Diseases of the Endocrine Glands, Williams & Wilkins, pp. 7–8.

15. Vogelaar, J. P. M. and Erlichman, E. 1936, Am. J. Cancer, 26: 358–367.

recently in growth and differentiation of embryonic skeletal tissue in vitro.[16] In the latter experiments explants of chick embryo limb buds showed acceleration of differentiation and sometimes of growth in vitro with l-thyroxin at concentrations of 0.01 to 0.16 μg/ml, but with the higher concentrations there was later inhibition followed by cellular degeneration. Although similar concentrations of thyroxin were used in our experiments, the early stimulatory effect on growth of chorioallantoic tissue, if it were present, could not be detected by microscopic examination. There seems, moreover, to be a disassociation between tissue proliferation and virus growth in these observations, since the amount of visible epithelium in the thyroxin-treated cultures tended to disappear between the 1st and 2nd day at a time when the hemagglutinins were still increasing.

The latent period of about 12 hours before the increase in respiration of tissue treated with thyroxin suggests a slow diffusion of the compound into cells and is quite different from the rapid stimulation of respiration observed with uncoupling agents, such as 2,4-dinitrophenol or the butyl ester of 3,5-di-iodo-4-hydroxybenzoic acid. If thyroxin exerts an uncoupling effect similar to the above mentioned compounds it is probably at a later period, after the first 24 hours, when the rate of virus synthesis slows down. This secondary effect, whatever its biochemical nature, seems to augment the damage to the tissue already containing large amounts of virus as indicated by the depressed respiration. The increased late autolysis of tissue in cultures containing both thyroxin and virus may be attributable to similar effects.

The substrate seemed to play a definite part in the observed effects of thyroxin on virus growth, since the stimulatory effects were most evident in mediums which were suboptimal, such as those with glutamate, pyruvate or no substrate. Glucose, which acts as a more suitable source of energy for virus synthesis in these tissue cultures, tended to obscure the stimulatory action of thyroxin on the virus, although the effects on respiration were the same. On the other hand, ATP and AMP, while tending to depress virus growth in the controls, resulted when combined with thyroxin in still further depression. We cannot at present offer any explanation in biochemical terms for the latter observation. They do suggest a connection between these substances and the thyroxin metabolism of intact cells, and it is possible that variations in the content of adenosine phopshate in different samples of tissue may account for the somewhat irregular stimulatory effects with thyroxin.

There was no evidence that virus inhibition could be brought about by higher concentrations of l-thyroxin. We observed no differences between the effects of l-thyroxin and l-tri-iodothyronine, except that the latter was stimulatory at lower concentrations as has been observed in experiments with other biological materials. Although studies have not yet been done on virus growth with d-thyroxin by itself, there is some evidence from our data with dl-thyroxin that this isomer may be inhibitory at concentrations above 10 μg/ml and may act, therefore, only as an uncoupling agent.[5]

No relation of Ca^{++} or K^{+} to the action of thyroxin on virus growth could be demonstrated, although Ca^{++} has been reported to depress and K^{+} to increase the stimulatory effect of thyroxin on growth of fibroblasts in tissue cultures.[15] Measurements of virus in tissue and supernatant fluids failed to reveal any effects of thyroxin on ad-

16. Fell, H. B. 1954, Ann. N. Y. Acad. Sci. **58**: 1183–1187.

sorption or liberation of virus, but the possibility of effects at the cell membrane has not been excluded.

Tissue cultures may furnish a useful system for the study not only of the effects of thyroxin on virus growth and tissue damage, but also of certain biochemical aspects of the mode of action of this hormone. The well known stimulatory effects of thyroxin and related compounds on metabolism of the intact animal have been difficult to demonstrate by adding these substances to isolated tissues or cell fragments in vitro.[17]

SUMMARY

L-Thyroxin added at 0.01 to 1.0 μg/ml to tissue cultures of chorioallantoic membrane of 11- to 12-day-old chick embryos produces an increase in oxygen consumption after a lag period of about 12 hours. No stimulatory effects on tissue growth were observed but inhibition of the extension of epithelium and fibroblasts was seen after 48 hours.

Slight stimulation of growth of influenza virus was observed in the presence of thyroxin, and in infected cultures with thyroxin late depression of respiration and accelerated destruction of tissue was evident.

The effects depended to some extent on the substrate used in the tissue cultures (glucose, pyruvate, glutamate, or no substrate).

Higher concentrations of l-thyroxin, up to 50 μg/ml, were not inhibitory to virus growth but irregular inhibition was seen with two preparations of dl-thyroxin.

Adenosine phosphates changed the effect of l-thyroxin on virus growth from one of stimulation to relative inhibition.

17. Lardy, H. A. and Feldott, G. 1951, Ann. N. Y. Acad. Sci. **54**: 636–648.

EXPERIMENTAL CANINE LEPTOSPIROSIS

I. *LEPTOSPIRA ICTEROHEMORRHAGIAE* INFECTIONS IN IMMATURE DOGS

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Acute leptospirosis in dogs usually occurs sporadically and under conditions which have made clinical, biochemical, and pathological changes difficult to correlate.[1] Experimental infection of dogs by inoculation with viable organisms has not, in the past, produced severe disease consistently enough to permit an adequate study of the acute-phase syndrome.[2,3] The present report describes attempts in this laboratory to establish a reproducible disease pattern in young dogs which might permit a detailed study of the acute phase and might serve as a basis for the evaluation of prophylaxis and therapy.

MATERIALS AND METHODS

Experimental animals.—A total of 73 dogs of mixed breeds and without selection as to sex were used in the course of this study, which was carried on over a period of 18 months. They ranged in age from 2 to 6 months and weighed from 5 to 21 pounds. Some of the dogs showed signs of distemper or upper respiratory tract infection after acceptance. In these instances, the control or experimental data obtained were discarded or appropriately qualified. Wherever feasible, data obtained from each dog were controlled with data from the same dog prior to infection. The serum of each dog was tested initially for leptospiral antibodies (against 16 serotypes) by the agglutination-lysis procedure.[4] None of the serums contained agglutinins detectable at a final dilution of 1:10 or greater.

Infectious agent.—The original source of the infectious agent was a laboratory stock culture of *Leptospira icterohemorrhagiae*, CF-1, in Fletcher's medium.[5] The culture was isolated in 1950 from a jaundiced dog in Puerto Rico.[6] The agent was modified in the course of the animal passages subsequently described. Recovery of the organism from infected animals was accomplished by direct inoculation of 5 ml portions of Fletcher's medium with 2 or 3 drops of whole blood. Cultures were incubated in the dark at 30 C and were not discarded as negative until 28 days had elapsed.

Biochemical methods.—Because of the small size of the dogs used in this study the amount of blood that could be taken at regular intervals necessarily limited the number of biochemical tests that could be performed. A number of relevant procedures were excluded for this reason; others were rejected because they were not applicable to young dogs or were unduly laborious for the amount of information afforded. Among the methods discarded were thymol turbidity, cephalin-cholesterol flocculation, prothrombin time, phenolsulfonphthalein excretion, acid and alkaline phosphatase, cholesterol, and erythrocyte fragility. The other procedures cited were

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1. Bloom, F. 1952, Symposium on the Leptospiroses, Army Med. Service Grad. School, Wash., D. C., 118–123.
2. Uhlenhuth, P., Schoenherr, K. E. and Zimmerman, P. 1950, Ztschr. f. Immunitätsforsch. **108**: 1–36.
3. van Riel, J., Schoenaers, F., Brouwers, J., Bienfet, V. and Kaekenbeeck, A. 1953, Rev. belge path. et med. exper. **23**: 38–53.
4. Gochenour, W. S., Jr., Yager, R. H., Wetmore, P. W. and Hightower, J. A. 1953, Am. J. Pub. Health, **43**: 405–409.
5. Fletcher, W. 1928, Trans. Roy. Soc. Trop. Med. & Hyg. **21**: 265–288.
6. Alexander, A. D., Wetmore, P. W., Evans, L. B., Yager, R. H. and Gochenour, W. S., Jr. To be published.

performed essentially as described by Simmons and Gentzkow,[7] except for bromsulfalein excretion, which was done by a spectrophotometric version of the method of Rosenthal and White,[8] and urinary urobilinogen, which followed the method of Watson.[9] Red cell survival rates were determined by a modification of the method of Ebaugh et al.[10]

EXPERIMENTAL AND RESULTS

Development of a virulent strain.—The series of dogs in which experimental infection was attempted may be divided into 8 successive groups. The members of the first group were inoculated with a heavy suspension of organisms collected from the growth zones of a stock culture of *Lept. icterohemorrhagiae*, CF-1. Each successive group was inoculated with organisms recovered from the preceding group, or with a saline suspension (20%) of macerated liver and kidney tissues from hamsters infected with organisms from the preceding group. Thus, in effect, the organism was cultivated through a series of 8 passages in the dog with intermediate culture in vitro and passage in the hamster. The details of this series of passages and the fortuitous increase in virulence which accompanied it are shown in table 1.

Before it became evident that the increasing virulence of the agent would be responsible for a frequent occurrence of severe symptoms, 15 dogs were rendered anemic by the daily withdrawal of blood with the hope that this stress would increase susceptibility to the disease. However, the anemic dogs did not differ from the normals in susceptibility or in severity of the response. There was, in fact, no apparent relationship between the manifestations of leptospirosis and the blood-depletion anemia.

Classification of response.—For convenience in assembling data, the infected dogs were classified by level of response according to the arbitrary criteria listed in table 2. Among 72 dogs infected, subclinical responses were observed in 20 dogs, mild responses in 18, moderate responses in 22, and severe responses in 12. The following clinical records were selected as typical of the various levels of response.

Subclinical response.—Dog 3583, a 13-pound male, was inoculated with a saline suspension of infected hamster tissue. A minor temperature elevation on the day of inoculation was regarded as insignificant. The organism was recovered from the blood on the 1st day after inoculation and for 2 days thereafter. No physical signs of illness were observed except for a mild tonsillitis on the 5th day. No gross abnormalities were noted at necropsy when the dog was sacrificed after 21 days. (See fig. 1.)

Mild response.—Dog 3582, a 21-pound female, experienced a significant rise in temperature on the 2nd day after inoculation. No other signs of illness were evident until the 5th day, when pharyngitis, inflamed and petechiated tonsils, and pain in the region of the kidneys were observed. The tonsillitis disappeared after the 10th day. The dog was alert, active, and ate well during the entire course of the disease. Leptospires were recovered from the blood for 4 days after inoculation. The animal was sacrificed on the 21st day. Gross findings at necropsy were confined to the kidneys. The renal capsules were adherent, and white fibrous areas about 2 mm in diameter were present

7. Simmons, J. S., Gentzkow, C. J. 1944, Laboratory Methods of the United States Army, ed. 5, Philadelphia, Lea & Febiger.
8. Rosenthal, S. M. and White, E. C. 1925, J.A.M.A. **84**: 1112–1114.
9. Watson, C. J. 1936, Am. J. Clin. Path. **6**: 458–475.
10. Ebaugh, F. G., Jr., Emerson, C. P. and Ross, J. F. 1953, J. Clin. Investigation **32**: 1260–1276.

TABLE 1.—*Development of a virulent culture of* Lept. icterohemorrhagiae *by passage through dogs and hamsters.*

Passage sequence	Total number of dogs	Number of dogs developing:				
		Febrile response	Leptospiremia*	Jaundice	Uremia	Fatal disease
1. Concentrated organisms from 8-day-old laboratory culture on Fletcher's medium inoculated IP and IV. Each dog received the organisms from 48 ml of culture.	2	1	1	1	0	0
2. Blood cultures on 1st to 5th days from both dogs in passage 1 were grown 14 days or more on Fletcher's medium. Concentrated organisms from 72 ml of culture inoculated IP and IV.	3	1	1	0	0	0
3. Blood from the sick dog in passage 2 was cultured and inoculated IP along with cultures from hamsters† infected with organisms concentrated from 42 ml of culture from passage 1.	4	4	3	1	0	0
4. Blood culture from 2 of the dogs in passage 3 was inoculated into hamsters. Pooled liver and kidney tissues from 11 hamsters were inoculated IP in equal doses.	4	3	2	2	2	1
5. Procedure of passage 4 repeated with tissues from 4 infected hamsters inoculated IP into each dog.	8	7	7	6	2	1
6. Cultures from the 2 uremic dogs of passage 5 inoculated into hamsters. After 3 hamster passages, dogs were inoculated with the hamster tissue, each dog receiving an equal amount of tissues from 28 1st passage hamsters and 9 3rd passage hamsters.	10	8	7	7	3	2
7. Cultures from the 2 dogs which died in passage 6 were inoculated into hamsters. First passage hamster tissues were inoculated IP. Each dog received liver and kidney tissues from 4 hamsters.	6	5	4	4	2	1
8. Culture from the dog which died in passage 7 was inoculated into hamsters. Tissues from four 2nd passage hamsters were inoculated IP into each dog.	4	4	4	4	3	0

The inoculum used in the red blood cell survival experiment was obtained from a later passage of the agent.
* Of 5 days duration or greater.
† Weanling hamsters 15 to 20 g in weight were used. Inoculation was by the intraperitoneal route.

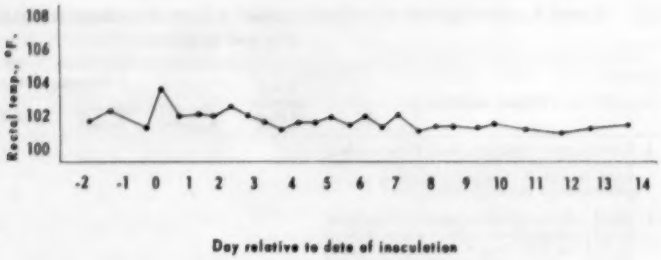
mainly on the subcapsular surface, although some of them extended halfway through the cortex. (See fig. 2.)

Moderate response.—Dog 3576, a 14-pound male, exhibited a slight febrile response of 12 hours' duration following inoculation with infected hamster tissue. Positive blood cultures were obtained for 5 days after inoculation. A slight and possibly nonspecific tonsillitis was noted during the first 4 days. Jaundice, bilirubinuria, and scleral injection were observed on the 5th day. The dog's appetite was poor, and the feces were noticeably lighter in color. There was a gradual remission of symptoms after the 7th day, although the plasma bilirubin remained elevated until the 15th day. The dog was sacrificed on the 26th day. At necropsy the gall bladder contained clear fluid bile. The liver was normal in size and consistency, but was

TABLE 2.—*Classification of responses of dogs following inoculation with* L. icterohemorrhagiae.

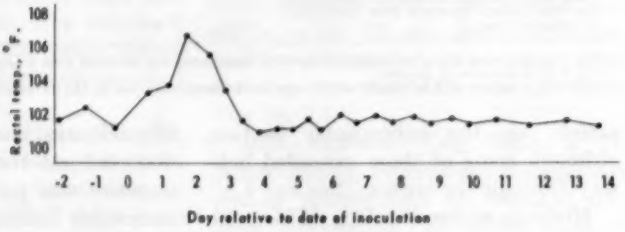
Response	Fever	Duration of leptospiremia*	Jaundice	Nitrogen retention
Subclinical	None	3 days or less	None	None
Mild	Present	3 to 5 days	None	None
Moderate	Present	5 days or more	Slight to moderate	Slight or none
Severe	Present	5 days or more	Severe	Moderate to severe

* Beginning, in all instances, on the 1st day after inoculation.



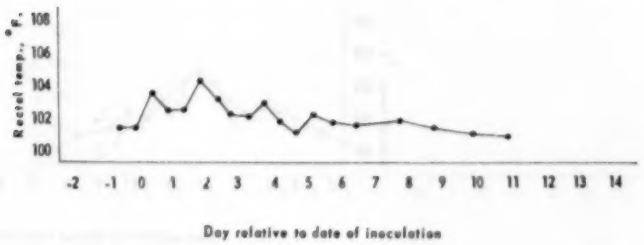
Hematocrit	40	36	37	35	35	34
Hemoglobin, gm%	13.0	11.2	12.7	11.6	11.1	11.1
Sedimentation rate, mm/hr	2	30	34	28	16	20
WBC, $10^3/mm^3$	15.2	20.0	17.0	11.2	20.0	18.5
Plasma bilirubin, mgm%						
Urinary bilirubin	- - - - - - - - - - - - - - - -					
Urinary urobilinogen, EU/2 hrs			0.19		0.31	0.25
Plasma uric acid, mgm%						
Bromsulfalein retention, %				1		
Blood urea nitrogen, mgm%	11.5		9.5	12.5		
Inorganic phosphorus, mgm%	7.6		7.6			

Fig. 1.—Dog 3583, subclinical response.



Hematocrit	38	39	36	32	35	36
Hemoglobin, gm%	12.3	12.1	11.8	10.8	11.1	11.0
Sedimentation rate, mm/hr		31	17	19	14	9
WBC, $10^3/mm^3$	12.2	16.9	11.2	9.0	14.8	10.6
Plasma bilirubin, mgm%						
Urinary bilirubin	- - - - - - - - - - - - -					
Urinary urobilinogen, EU/2 hrs		0.50	0.75			
Plasma uric acid, mgm%						
Bromsulfalein retention, %				0		
Blood urea nitrogen, mgm%	14.0		15.0	14.5		
Inorganic phosphorus, mgm%						

Fig. 2.—Dog 3582, mild response.



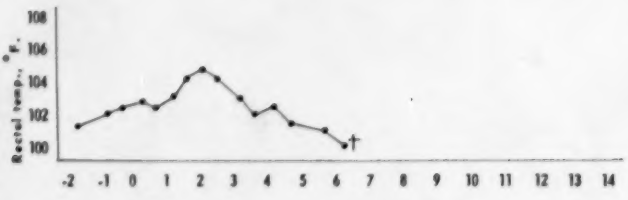
Hematocrit	40			34			35		34								38
Hemoglobin, gm%	11.4			10.2			10.8		9.5								11.4
Sedimentation rate, mm/hr	1			24			43		21								20
WBC, $10^3/mm^3$	10.4			6.5			20.6	34.9	27.0								26.0
Plasma bilirubin, mgm%							1.5										
Urinary bilirubin	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+
Urinary urobilinogen, EU/2 hrs	0.39	0.28					0.30		0.46		0.37						
Plasma uric acid, mgm%		0.67					1.4										
Bromsulfalein retention, %							10										
Blood urea nitrogen, mgm%	10.0						20.0	26.5	29.5		16.0						
Inorganic phosphorus, mgm%											6.9						

FIG. 3.—Dog 3576, moderate response.

distinctly lobulated. White fibrous areas about 2 mm in diameter were scattered over the subcapsular surfaces of the kidneys. Old ecchymotic hemorrhages were noted in the lungs. (See fig. 3.)

Severe response.—Dog 3575 was an 8-pound female, 3 months old at the time of inoculation. Blood cultures were positive for the first 5 days and were not attempted thereafter. Jaundice and bilirubinuria, first observed on the 4th day, became progressively more severe with time. On the 5th day, severe depression was observed. Petechial hemorrhages were present on the tonsils, and a watery nasal discharge was noted. The dog refused all food. The feces were light grey in color. On the 6th day the dog was dehydrated and very weak. Severe jaundice and scleral injection were present. Death occurred on the 7th day. At necropsy, a marked jaundice involving the skin, mucous membranes, serous surfaces of the organs, cartilages, and fat was observed. Bloody mucus was present around the anus. The liver was friable, yellow-brown in color, and distinctly lobulated, but not abnormal in size. The gall bladder was completely empty. The kidneys were pale yellow-brown in color with numerous subcapsular petechial hemorrhages. The renal capsules were not adherent. The left kidney was slightly swollen. The mucosa of the small intestine was congested and covered with an excessive quantity of mucus. An intussusception, apparently of short duration, was found in the ileum, and free blood was observed in the bowel posterior to the intussusception. Ecchymotic hemorrhages were present throughout the lungs. (See fig. 4.)

Dog 3597, an 8-pound female, had been rendered anemic by daily withdrawal of blood for a period of one month. With the exception of the induced anemia, the animal was in ap-



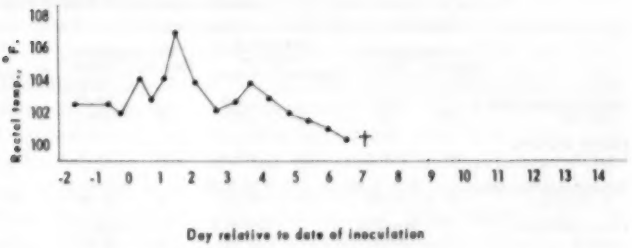
Hematocrit	36		35		31			
Hemoglobin, gm%	11.0		10.4		10.5			
Sedimentation rate, mm/hr			37		38			
WBC, $10^3/mm^3$	12.9		9.3		13.6			
Plasma bilirubin, mgm%								7.3
Urinary bilirubin	-	-	-	-	-	+	+	+
Urinary urobilinogen, EU/2 hrs								
Plasma uric acid, mgm%								
Bromsulfalein retention, %								
Blood urea nitrogen, mgm%	10.0				44.5			
Inorganic phosphorus, mgm%		7.3			12.6			

FIG. 4.—Dog 3575, severe response.

parent good health at the time of inoculation, having gained 4 pounds during the bleeding period. Vomiting and transient depression occurred after inoculation. Positive blood cultures were obtained for 6 days. During the first 3 days, the dog was active, alert, and had a good appetite. Jaundice, bilirubinuria and scleral injection were observed on the 4th day. Jaundice and bilirubinuria increased during the following days. On the 5th day dehydration was evident, the feces were blood-tinged and vomition was frequent. By the 6th day, dehydration and weakness were extreme. The nostrils were occluded with a sanguinous discharge. The animal, in a moribund condition, was sacrificed on the 7th day. At necropsy the intense and widespread jaundice was a striking observation. The liver was yellow-brown in color with distinct lobulation, and the gall bladder was empty. The kidneys were yellow-brown in color with numerous petechial hemorrhages underlying their capsules. A hemorrhagic area 4 cm long and 3 cm wide was present in the wall of the small intestine. Acholic feces were present in the rectum. Ecchymotic hemorrhages were found throughout the lungs. (See fig. 5.)

General clinical observations.—The administration of infected hamster tissues commonly resulted in a mild febrile reaction not associated with leptospirosis. The same febrile reaction occurred in control animals receiving normal hamster tissues. This reaction occurred within a few hours after inoculation and did not exceed 12 hours in duration. A local reaction, consisting of slight abdominal tenderness for 2 days, was noted in only 6 of the inoculated dogs.

The first indication of disease was a febrile response on the 2nd or 3rd day, with temperatures rising to 104.0° to



Hematocrit	21		20		23			24		
Hemoglobin, gm%	7.3		5.5		6.3			6.5		
Sedimentation rate, mm/hr	9		9		40			24		
WBC, $10^3/mm^3$		18.9			6.8			20.3		
Plasma bilirubin, mgm%									9.7	12.5
Urinary bilirubin	–	–	–	–	–	–	+	+	+	+
Urinary urobilinogen, EU/2 hrs					0.38					
Plasma uric acid, mgm%										
Bromsulfalein retention, %										
Blood urea nitrogen, mgm%			6.5					66.5		114.5
Inorganic phosphorus, mgm%			7.4							17.7

FIG. 5.—Dog 3597, severe response.

106.5 F. The duration of hyperthermia was approximately 36 hours. Of the dogs inoculated with material in the first 5 passages (see table 1), 76% demonstrated febrile responses. With the more virulent organisms used in the last 4 passages the frequency of the febrile response increased to 87%. Usually fever was the only early sign of illness. Dogs with temperatures as high as 106 F often remained active, alert and ate well.

Other manifestations of illness, beginning on the 4th day after inoculation, consisted of depression, partial anorexia, pharyngitis, tonsillitis (often with petechial hemorrhages) and pericorneal injection. Jaundice, when it occurred, was evident in the sclera on the 4th or 5th day. Bilirubinuria regularly preceded visible jaundice. Scleral hemorrhages were occasionally observed. An abrupt change in fecal pigmentation was a regular occurrence in jaundiced animals, the feces sometimes changing in color from brown to gray within a single bowel movement.

As the disease progressed, jaundice became apparent in the skin and mucous membranes, and vomiting and diarrhea or constipation were frequently observed. Bloody feces were passed occasionally, but blood loss in these instances was negligible. Mucoid, mucopurulent, or sanguinous nasal discharges were common and were sometimes distinctly stained with bile. Coughing often occurred in the severe cases.

Some of the dogs displayed tenderness in the region of the kidneys, but this symptom did not correlate with perceptible renal damage. The liver was not palpable in any of the dogs. Dogs suffering severe infections displayed complete anorexia, dehydration, and extreme weakness.

In dogs which survived the acute phase with moderate or severe re-

TABLE 3.—*Laboratory data tabulated according to level of response obtained**

	Normal control		Subclinical response		Mild response		Moderate response		Severe response	
	Number	Value	Number	Value	Number	Value	Number	Value	Number	Value
Sedimentation rate, mm/hr	22	0–7 (2)	8	4–39 (21)	10	10–57 (31)	9	8–68 (36)	6	28–75 (54)
Plasma bilirubin, mg %	69	<0.5	22	<0.5	18	<0.5	10	0.8–7.1 (3.3)	9	1.7–20.8 (9.0)
Urinary urobilinogen E. U./2 hrs.	10	0.21–0.40 (0.30)	2	0.19–0.22 (0.21)	2	0.50–0.62 (0.56)	5	0.23–0.55 (0.41)	2	0.33–0.38 (0.35)
Plasma uric acid, mg %	7	0.5–0.8 (0.64)	2	0.5–0.7 (0.61)	4	0.8–1.0 (0.86)	3	1.2–2.2 (1.58)	1	1.9
Bromsulfalein retention %	27	0–3 (<1)	15	0–4 (1.2)	11	0–11.5 (3.5)	13	0–24 (10.3)	5	0–25 (18.1)
Blood urea nitrogen, mg %	53	6–22 (10.9)	5	9–13 (10.4)	13	7–21 (9.3)	18	8–38 (16.9)	9	30.0–173 (79.8)
Inorganic phosphorus, mg %	12	6.2–9.6 (7.4)	3	7.2–8.7 (7.8)			8	4.9–10.9 (7.6)	5	12.6–19.4 (15.5)

Blood samples were drawn in the morning, 18 hours after offering food.
* The range of values is shown, with the mean values in parentheses.

sponses, evidence of recovery was often apparent on the 8th day. After the 10th day the residual symptom was a fading jaundice which persisted as long as 4 weeks.

Biochemical data.—The results of the biochemical tests performed are shown in table 3, arranged according to the level of response. Normal data consist of values obtained for experimental dogs prior to inoculation or from control dogs during the course of the experiments. The entries under the various levels of responses are the extreme values obtained for individual dogs. In almost every instance, the extreme value was that reported on the 5th to 7th day. The table, therefore, represents the values obtained at or near the peak of response.

An increased sedimentation rate was observed on the 2nd day after inoculation, even in those dogs with subclinical responses. The maximal rate usually occurred on the 7th to 10th day in surviving animals.

Plasma bilirubin was invariably of the direct reacting type. The peak of bilirubinemia in dogs which eventually recovered occurred on the 6th to 8th day, followed by a slow and regular reduction. Dogs which died or were sacrificed in a moribund state showed a constant increase in plasma bilirubin from the onset of jaundice until the day of death. Bilirubin appeared in the urine prior to its detection in the plasma and, in surviving animals, disappeared from the urine several days before plasma bilirubin levels returned to normal. When the dogs were observed in bright daylight the appearance of jaundice in the sclera was nearly as sensitive as plasma bilirubin level as an indication of the onset of icterus. Visible jaundice was observed in one dog with a plasma bilirubin concentration of only 0.8 mg%.

Urinary urobilinogen excretion did not change markedly from preinoculation values in any of the few dogs studied in this respect. Only two satisfactory measurements of urinary urobilinogen in severe leptospirosis were made. Other attempts to perform the test were unsuccessful because of fecal contamination of the sample or an insufficient quantity of urine. Plasma uric acid levels paralleled the development of jaundice, and remained within normal limits when jaundice did not occur. Bromsulfalein retention displayed a similar parallel relationship, although retentions up to 10% were noted in several infected animals which did not

become icteric. With this exception, in the absence of jaundice, the liver function tests used consistently gave values with the accepted normal ranges. Thus, in the experimental disease in dogs, the plasma bilirubin level appeared to be the most reliable index of hepatic impairment. Bromsulfalein retention was zero in one dog in which the test was performed 24 hours prior to the onset of visible jaundice.

Some degree of nitrogen retention was observed in the moderate or severe infections. The elevation in blood urea nitrogen was frequently apparent on the 4th day after inoculation. Among 45 infected dogs tested, 14 had definite nitrogen retention, and an additional 5 probably had slight retention. Blood urea nitrogen concentrations above 25 mg% were considered to be significant of loss of renal function in consideration of the relatively low levels (6 to 22 mg %) observed in the uninfected dogs. Rapid increases in blood urea nitrogen concentration in most instances were followed by a moribund state. In 2 dogs however, blood urea nitrogen levels decreased and approached pre-inoculation values before death occurred. Inorganic phosphorus determinations on a few of the infected animals indicated that phosphorus retention and nitrogen retention were essentially concurrent.

Hematological observations.—A decrease in hemoglobin concentration amounting to 1 to 2 g% with a corresponding reduction in hematocrit was often observed during the first several days after inoculation. The observations suggested an increased rate of red blood cell destruction, although neither hemoglobinemia nor hemoglobinuria was ever observed in any of the animals.

In an experiment to determine the rate of red blood cell destruction in infected dogs, the erythrocytes of 4 young dogs were labeled with ^{51}Cr. Two days later, 3 of the dogs were inoculated with infected hamster tissue. Two ml blood samples were withdrawn every other day from each dog. The 3 infected dogs became jaundiced, and 2 of them died on the 5th day. The erythrocyte survival, computed as percent of original radioactivity per gram hemoglobin, is shown in table 4. The jaundice in these animals was not accompanied by any demonstrable increase in red blood cell destruction rate.

White cell counts fluctuated considerably in the animals. In certain of the infected dogs a transient leucopenia occurred at the time of the febrile response. Leucocytosis occurred regularly as a delayed phenomenon, seldom becoming apparent before the 5th day after inoculation. White cell counts as high as 35,000/mm³ were recorded after the 5th day.

Bacteriological observations.—Viable leptospires were recovered from the blood of 68 of 70 (97%) of the dogs in which isolation was attempted. Blood cultures were taken on the day after inoculation and on each day thereafter for about 10 days. The duration of leptospiremia ranged from 2 to 8 days and appeared to correlate with the severity of the clinical symptoms. No systematic attempt was made to recover leptospires from the urine. Serological examination of 41 serums from infected

TABLE 4.—*Survival of red cells in 1 normal and 3 infected dogs.*

Hours after tagging	Percent red cells surviving			
	Dog 207 (normal)	Dog 208 (infected)	Dog 209 (infected)	Dog 210 (infected)
0.25	95.5	97.3	97.1	94.8
44	81.3	80.0	82.7	79.2
66	75.7	77.0	79.8	76.2
144	65.7	67.2	73.2	66.5
187	65.1	64.2	69.7	dead
235	60.8	dead	67.9	
307	52.9		60.7	
355	51.2		55.2	
403	48.4		53.2	
Half-life, days	20	20	25	20

dogs revealed specific agglutination-lysis titers ranging from 1:40 to 1:24,600, with no evident correlation with the severity of the disease or the duration of leptospiremia. The highest titer occurred in a dog which had a subclinical response and leptospiremia of only 3 days duration.

Gross pathology.—Seven dogs succumbed to the disease or were sacrificed in an obviously moribund condition within 6 to 10 days after inoculation. Ecchymotic hemorrhages of the lung were observed in 6 of these dogs, and 2 had free blood in the bronchi. The liver was friable in 2 dogs, and exaggerated lobulation was observed in 3. A yellow-brown coloration was present in the livers of all 7 dogs. No alterations in size were apparent, but the livers were not weighed and small variations would not have been detected.

Two dogs had no bile in either the bile duct or the gall bladder; the other 5 had inspissated bile in the gall bladders although the extra-hepatic bile ducts were empty.

In 4 of the dogs, blood was found in the colon and rectum. One dog had a recent intestinal intussusception. Acholic feces were present in the large intestines of all dogs. Swollen kidneys were observed in 5 of the 7 dogs, and a yellow-brown coloration was uniformly present. The kidneys in one dog had a mottled appearance, and in another the renal capsule was adherent. Petechial hemorrhages were found on the renal surfaces in 2 dogs. No gross lesions of the heart, spleen, pancreas, or adrenals were noted.

Fifty additional dogs were sacrificed after physical signs of leptospirosis were no longer evident. No significant lesions were found in 13 of these animals. Ecchymotic hemorrhages of the lungs were observed in 4 animals. No liver abnormalities were noted except for exaggerated lobulation in 6 dogs. In animals which had been jaundiced a dark residue was found in the gall bladder. A large mass of solidified bile was present in the gall bladder of one dog which was sacrificed 22 days after inoculation.

Small white areas 2 mm in diameter were seen on the subcapsular surfaces of the kidneys in 11 of the animals. These areas were usually confined to the surface, but some extended through the cortex to the medulla. The renal capsules were adherent in 16 of the 50 dogs. Bulging of the cut surfaces of the kidneys was observed in 2 animals, and the kidneys of 2 others appeared mottled. Petechial hemorrhages were noted in the kidneys of 4 dogs. No gross abnormalities of the heart, gastro-intestinal tract, adrenals, spleen, or urinary bladder were observed in any of the recovered animals.

DISCUSSION

Since experimental infection by means of parenteral inoculation with a large number of viable leptospires differs widely from the natural mode of infection, one would expect certain differences between the experimental disease and its natural counterpart. A prominent difference observed was a shortening of the incubation period from the 4 to 12 days which is characteristic of the natural disease[11,12] to 3 days or less in the experimental infection.

Comparison of the experimental and natural diseases requires recognition of the fact that natural infections at the subclinical or mild levels are likely to escape detection and are therefore not adequately represented in published re-

11. van Thiel, P. H. 1948, The Leptospiroses, Univ. Pers Leiden, p. 95.
12. Schüffner, W. 1934, Tr. Roy. Soc. Trop. Med. & Hyg. **28**: 7–37.

ports of the natural disease in dogs. Klarenbeek[13] in 1938 reported observations on 151 young dogs with confirmed leptospirosis, including both *L. canicola* and *L. icterohemorrhagiae* infections. In 7% of these dogs the disease was recognized without either icterus or uremia. Icterus occurred in 82% of the cases, uremia in 21%, and concurrent icterus and uremia in 10%. The occurrence of uremia without jaundice in 11% of these dogs is in contrast with the results of the present study, in which jaundice was an invariable antecedent of measurable renal impairment. The regularity of jaundice as a symptom of severe disease in the experimental cases may have resulted from a characteristic property of the pathogen, or it may have been a consequence of the massive parenteral inoculation.

Leptospiral jaundice in cattle has been described by Reinhard[14] as primarily hemolytic in origin, and this conclusion is supported by the frequency with which hemoglobinuria occurs. In human leptospirosis, on the other hand, the jaundice appears to result principally from damage to the parenchymal cells of the liver,[15] although it may be aggravated by obstruction of the bile canaliculi.[16]

It seems probable that increased red cell destruction is not a factor of importance in the experimental infection in dogs. The major cause of jaundice appears to be an impairment of excretion of crystalloidal bilirubin from the liver. Some of the excretory impairment may be due to intrahepatic biliary stasis, as observed by Olafson.[17] Additional information will be available when histopathological studies in progress have been completed.

Severe jaundice in the infected dogs was usually accompanied by evidence of renal insufficiency. The blood urea nitrogen concentration on the 5th day after inoculation was an accurate prognostic index, indicating that the fate of the animal depended upon the extent of renal involvement. Kidney function studies have been performed on many of the experimental dogs. The results, summarized in a later publication in this series,[18] afford a more detailed view of the nature of the renal impairment.

SUMMARY

Inoculation of immature dogs with *Leptospira icterohemorrhagiae*, CF-1, produced acute infections with symptoms classified as subclinical (28%), mild (25%), moderate (31%), or severe (17%). Jaundice, which occurred in 47% of the dogs, appeared to be due to hepatocellular injury. In severe or fatal disease, jaundice was accompanied by uremia. Clinical, biochemical, and gross pathological changes characteristic of the several levels of response have been described.

13. Klarenbeek, A. 1938, Acta Conv. Tert. de Trop. Morbis **1**: 381–386.
14. Reinhard, K. R., 1952, Symposium on the Leptospiroses, Army Med. Service Grad. School, Wash., D. C., 126–139.
15. Koppisch, E. and Bond, W. M. 1952, Symposium on the Leptospires, Army Med. Serv. Grad. School, Wash., D. C., 83–105.
16. Sheehan, H. L. 1946, Brit. Med. J. **1**: 83.
17. Olafson, P. 1953. Paper presented at the 3rd Gaines Veterinary Symposium, Gaines Dog Research Center, 250 Park Ave., New York City, p. 14–15.
18. Low, D. G., Bergman, E. N., Hiatt, C. W. and Gleiser, C. A. 1956, J. Infect. Dis. **98**: 260–265.

EXPERIMENTAL CANINE LEPTOSPIROSIS

II. RENAL FUNCTION STUDIES

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Localization of leptospires in the kidney has long been recognized as a characteristic component of canine leptospirosis.[1] Recent studies by McIntyre and Stuart[2] and McIntyre and Montgomery[3] have emphasized the importance of *Leptospira canicola* infection as the cause of acute and chronic interstitial nephritis in dogs. Although the uremic syndrome has been more closely associated with *Lept. canicola* infections,[4,5] It has also been observed in dogs infected with *Lept. icterohemorrhagiae*.[6]

An earlier publication from this laboratory[7] described the clinical and biochemical data accumulated in the study of 73 dogs in which experimental *Lept. icterohemorrhagiae* infections had been established. Blood urea nitrogen concentrations were determined at frequent intervals in dogs exhibiting clinical evidence of disease. Significant impairment of renal function was implied in the more severely affected animals by marked increases in blood urea nitrogen as early as the fourth day after inoculation. Renal function tests performed on experimental and control animals in this group illustrate the extent of functional renal impairment that can occur in the acute phase of canine leptospirosis.

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1. van Thiel, P. H. 1948, The Leptospiroses, Universitaire Pers Leiden, 231 pp.
2. McIntyre, W. I. M. and Stuart, R. D. 1949, Vet. Rec. **61**: 411–418.
3. McIntyre, W. I. M. and Montgomery, G. L. 1952, J. Path. Bact. **64**: 145–160.
4. Dhonut, C. M., Klarenbeek, A., Schuffner, W. and Voet, J. 1934, Nederl. tijdschr. v. geneesk. **78**: 5197–5208.
5. Rosenberg, B. L. 1951, Am. J. Med. **11**: 75–91.
6. Klarenbeek, A. 1938, Acta. Conv. Tert. de Trop. Morbis, **1**: 381–386.
7. Low, D. G., Hiatt, C. W., Gleiser, C. A. and Bergman, E. N. 1956, J. Infect. Dis. **98**: 249–259.

MATERIALS AND METHODS

Experimental animals.—The experimental dogs used were part of a group of 73 dogs previously described.[7] They were dogs of mixed breed, without selection as to sex, and ranged from 2 to 6 months in age. The response to experimental infection was categorized for each animal as subclinical, mild, moderate, or severe, on the basis of the criteria previously established.[7] In all of the dogs in which the response was classified as "severe," an appreciable impairment of kidney function was indicated by significant and sustained elevations of blood urea nitrogen (BUN). In certain of the dogs in which the response was classified as "moderate," slight or transient elevations in BUN were observed.

Infectious agent.—Infection was established by intraperitoneal inoculation of saline suspensions of liver and kidney tissues from hamsters infected with serial passages of *Lept. icterohemorrhagiae*, CF-1. The enhancement of virulence resulting from passage of the agent in a dog-culture-hamster-dog sequence has been described previously.[7]

Kidney function tests.—All experiments were performed on dogs in a fasting state. About 45 minutes before each experiment, a stomach tube was introduced and a water "prime" of 50 ml/kg body weight was administered. Light anesthesia was established by intravenous administration of sodium pentobarbital and was maintained throughout the experiment. Blood samples for analysis were obtained through a polyethylene catheter (Clay Adams PE 50) inserted into either the right or left saphenous vein,

Insertion of the catheter into the vein a distance of 6 inches was required to be assured of adequate blood samples. Blood coagulation was retarded by wetting the catheter with heparin before introduction. A Luer-Lok coupler attached to the external end of the catheter permitted the use of a syringe to obtain blood samples as desired. A fitted Teflon plug was used to seal the end of the catheter between samples. The blood samples taken were confined to a volume of 4 to 6 ml in view of the small size of the dogs. After each sample had been taken, a small amount of heparin was injected into the catheter to prevent clotting and to serve as an anticoagulant for the next blood sample. The slight dilution error (1 to 2%) caused by the heparin solution was ignored.

A similar catheter was inserted into one of the external jugular veins to permit infusion of the test solutions. The sustaining solutions were administered at a constant rate (1 or 2 ml/min) by means of a mercury gravity pump calibrated at the beginning of each experiment.

A human ureteral catheter (4 to 8 Fr.) was employed for urine collection. The internal end of this catheter was positioned carefully so that a measured volume of saline solution injected into the empty bladder could be recovered promptly without significant loss. A blunt hypodermic needle forced into the lumen of the catheter at the external end was used to couple a syringe to the catheter for aspiration of urine and for flushing the bladder with saline solution and air at the termination of each collection period. The insertion and manipulation of this catheter was accomplished with care to avoid traumatic injury to the bladder mucosa.

The procedures for conducting the function tests followed established methods[8,9] with certain modifications in technique. Glomerular filtration rate was estimated by exogenous creatinine clearance, and sodium para-aminohippurate was used to determine effective renal plasma flow and tubular maximum (Tm_{PAH}). A priming solution containing appropriate quantities of creatinine and PAH was introduced and followed immediately by the start of the first sustaining solution. An equilibrium period of at least 20 minutes was allowed, then the bladder was emptied and washed and timed collection periods were started. The collection periods, usually 15 minutes in duration, were timed with a stopwatch. Blood samples were taken at the start and completion of each collection period. The bladder contents were aspirated 1 to 2 minutes before the end of each period, and the final minute was used for flushing the bladder with saline solution and air to insure complete collection. Two consecutive periods were allowed for determination of effective renal plasma flow. A second priming solution of PAH, sufficient to raise the blood concentration to Tm levels, was then introduced. The flow of a second sustaining solution, containing PAH in sufficient concentration to maintain Tm levels, was started immediately. Again an equilibrium period of at least 20 minutes was allowed, followed by two 15-minute collection periods. The Tm_{PAH} was considered valid[9] whenever the quantity of PAH presented to the kidney tubules was at least twice the measured Tm_{PAH}. Creatinine clearance was determined for all four collection periods.

Plasma, separated from the blood samples by centrifugation immediately after collection, was treated with 10% trichloracetic acid to precipitate the proteins. Analyses were performed on the protein-free plasma filtrates. Urine specimens for each period, pooled with the saline washes, were diluted volumetrically to 50 or 100 ml and then further diluted as necessary for the analyses. The final urine dilution was treated in exactly the same way as the plasma. The determination of PAH was conducted by a modification of the method of Brun.[10] Creatinine was determined by a spectrophotometric modification of the Folin-Wu method[11] using the Jaffe reagent.

EXPERIMENTAL RESULTS

Kidney function tests were performed on 17 dogs* prior to inoculation. The observed values for glomerular filtration rate, effective renal plasma flow, and tubular maximum for para-aminohippuric acid (Tm_{PAH}) in this group of essentially normal young dogs are shown in table 1. Repeated determinations on 3 of the dogs indicated that variations

8. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B. and Grabor, M. 1945, J. Clin. Investigation, **24**: 388–404.
9. Chasis, H., Redish, J., Goldring, W., Ranges, H. and Smith, H. 1945, J. Clin. Investigation, **24**: 583–588.

* Five of these dogs were anemic as the result of regular blood depletion.[7] The values obtained for these dogs did not appear to differ from those of the nonanemic dogs sufficiently to justify incorporation of a separate group. Anemic dogs are designated in table 1.

10. Brun, C. 1951, J. Lab. & Clin. Med. **37**: 955–958.
11. Folin, O. and Wu, H. 1919, J. Biol. Chem. **38**: 87.

TABLE 1.—*Kidney function tests of normal immature dogs prior to infection.*

Dog no.	Sex	Body weight kg	Glomerular filtration rate, ml/min/kg†	Effective renal plasma flow ml/min/kg‡	Tubular maximum (PAH) mg/min/kg‡
8	M	4.1	4.3	15.5	
3506	F	7.3	4.1	8.8	
3508*	M	6.4	3.2	8.2	0.54
3515	M	5.0	4.3	15.0	0.99
3519	F	3.2	3.9	10.9	
3522	F	5.9	4.4	9.9	0.88
(13 days later)		5.9	4.3	10.0	0.89
3532	F	4.5	3.6	10.5	
(12 days later)		5.5	4.9	12.4	0.76
(28 days later)		6.8	4.4	11.6	0.98
3542	F	5.5	5.2	13.7	
3543*	F	4.5	4.7	11.9	0.72
3563	F	5.0	4.8	15.5	1.03
3572*	F	6.1	4.0	8.9	0.56
3579	M	5.7	4.2	8.2	0.53
3581	M	4.5	3.5	7.8	0.85
(11 days later)		5.5	4.3	11.4	0.82
3594	F	3.6	4.3	11.5	0.55
3595	F	4.3	3.4	9.7	0.70
3597*	F	4.1	4.0	10.2	0.87
3598	M	4.6	4.4	13.5	
Mean(m)		5.14	4.20	11.20	0.778
		1.06	0.50	2.37	0.144
$\frac{100\sigma}{m}$		20.6	11.9	21.2	18.5

* Anemic.
† Mean of values for 4 consecutive periods.
‡ Mean of values for 2 consecutive periods.

of the order of $\pm 20\%$ can occur in ostensibly normal growing puppies.

In order to obtain a representative number of animals in each of the several response categories, it was necessary to expand the experimental group after infection was established to include 13 additional dogs for which pre-inoculation data were incomplete. Postinfection data were obtained from 8 dogs with mild or subclinical responses, 10 with moderate responses, and 5 with severe responses.

Table 2 presents the data obtained from infected dogs exhibiting mild or subclinical response to infection. The dogs which did not become icteric or uremic were previously classified[7] as subclinical cases (in the absence of fever) or mild cases (where a febrile response was noted). There was no significant change in the values obtained for glomerular filtration rate, effective renal plasma flow, or Tm_{PAH} as a result of subclinical or mild disease in any of the 8 dogs tested.

Kidney function tests on 9 dogs classified as having moderate responses

TABLE 2.—*Kidney function in dogs with mild or subclinical infections.*

Dog no.	Sex	Body weight kg	Days relative to date of inoculation	Glomerular filtration rate, ml/min/kg	Effective renal plasma flow, ml/min/kg	Effective renal blood flow ml/min/kg	Tm_{PAH} mg/min/kg	Filtration fraction	C_{PAH}/Tm_{PAH}
688	F	6.8	+10	3.3	14.3		0.85	23	16.8
3506	F	7.3	0	4.1	8.8	12.0		47	
		7.7	+18	3.9	10.2		0.72	38	14.2
3511	M	2.8	+14	5.4	13.8		0.85	39	16.2
3522	F	5.9	−13	4.4	9.9		0.88	45	11.2
		5.9	0	4.3	10.0		0.89	43	11.2
		5.9	+ 7	4.0	10.4	16.3	0.77	39	13.5
		6.1	+14	3.6	9.8	14.9	1.02	40	9.6
3532	F	4.5	−28	3.6	10.5	15.2		34	
		5.5	−16	4.9	12.4	17.4		40	
		6.8	0	4.4	11.6		0.98	38	11.8
		8.2	+ 7*	4.6	13.5	26.0	1.26	34	10.7
		8.6	+14	4.0	12.3		1.06	33	11.6
3542	F	5.0	0	5.2	13.7	22.0		38	
		5.5	+ 7	4.2	12.3	19.8	0.76	34	16.2
		5.5	+14	3.8	10.8	16.1	0.91	35	11.9
3579	M	5.7	0	4.2	8.2	12.4	0.53	51	15.5
		6.4	+ 7	4.2	10.4		0.96	40	10.8
		6.8	+14	4.6	12.9	19.4	0.94	36	13.7
3581	M	4.5	−11	3.5	7.8	11.4	0.85	45	9.1
		5.5	0	4.3	11.4	15.8	0.82	38	13.9
		7.3	+ 7	4.7	17.0	24.3	1.11	28	15.4
		7.5	+14	3.6	11.0	16.4	0.90	33	12.2

* Febrile during this determination.

TABLE 3.—*Kidney function in dogs with moderate infections.*

Dog no.	Sex	Body weight kg	Days after inoculation	Blood urea nitrogen mg %	Glomerular filtration rate, ml/min/kg	Effective renal plasma flow, ml/min/kg	Tm_{PAH} mg/min/kg	Filtration fraction	C_{PAH}/Tm_{PAH}
3504	M		0	10					
			5	11					
		2.3	7	10	5.0	13.4	0.78	37	17.2
			10	9					
3507*	M		0	14					
			5	8					
			9	14					
			12	14					
			16	12					
		4.3	26		3.4	11.7	1.18	29	9.9
3543*	F	4.5	0	8	4.7	11.9	0.72	40	16.5
			5	7					
			6	10					
			7	8					
			9	8					
			12	11					
		4.1	23		4.8	12.0	1.07	40	11.2
3544	M	2.6	7		4.4	12.8	0.51	34	25.1
			9	22					
3561	M	5.5	21		3.9	13.6		29	
3564	F		0	10					
			5	10					
			6	13					
		4.6	7	23	1.8	5.9	0.25	31	23.6
			9	30					
			12	12					
			16	15					
		3.6	19		3.5	13.6	0.52	26	26.2
		4.1	29		3.2	12.5	0.47	26	26.6
3572*	F	6.1	0	15	4.0	8.9	0.56	45	15.9
			5	12					
			6	9					
			7	8					
			12	12					
			16	18					
		5.0	20		3.7	8.5	0.32	44	26.5
3576	M	6.4	0	10					
			5	20					
			6	26					
			7	30	2.8	11.0	0.40	25	27.5
			9	16					
3599*	F		0	10					
			5	18					
			7	16					
		6.1	8		2.6	7.9	0.32	33	24.6
		6.4	22		4.3	13.0	0.62		
2	M		0	9					
			8	32					
		12.3	23		2.7	11.1		24	
		13.6	83		3.0	10.7		28	
		15.9	142		3.1	9.6	0.88	32	10.9
		13.6			3.7	12.6	1.10	29	11.4

* Dogs rendered anemic by daily withdrawal of blood.

to infection are summarized in table 3. Fever and jaundice were observed in all members of this group but nitrogen retention did not occur or was relatively slight. In those dogs in which no demonstrable nitrogen retention occurred, the kidney function data resembled the normal values reported in table 1. Preinoculation data for the dogs in which slight uremia occurred were unfortunately not available for comparison. However, in two instances (dogs 3564 and 3599) the function tests were repeated after apparent clinical recovery, and substantial increases in glomerular filtration rate, effective renal plasma flow, and Tm_{PAH} were observed.

Table 4 is a summary of the kidney function data obtained from 5 dogs with severe responses to infection. Extreme depression of glomerular filtration rate, effective renal plasma flow, and Tm_{PAH}

TABLE 4.—*Kidney function in dogs with severe infections.*

Dog no.	Sex	Body weight kg	Days after inoculation	Blood urea nitrogen mg %	Glomerular filtration rate, ml/min/kg	Effective renal plasma flow, ml/min/kg	Tm_{PAH} mg/min/kg	Filtration fraction	C_{PAH}/Tm_{PAH}
3575	F		0	10					
		3.6	5	44	0.4	0.8	0.04	50	20.0
3590	F		0	6					
			5	8					
			6	13					
		3.4	7	34	1.8	3.9	0.26	46	15.0
			9	20					
			12	12					
			16	16					
3595	F	4.3	0	8	3.4	9.7	0.70	35	13.8
		3.9	5	86	0.8	2.1	0.04	38	52.5
			6	70					
3597*	F	4.1	0	6	4.0	10.2	0.87	39	11.7
			5	66					
		3.4	6		0.3	0.8	0.04	38	20.0
			7	114					
3905*	F		0	16					
		5.5	4	64	1.0	3.9	0.14	26	27.8
		5.0	7	32	1.3	6.2	0.30	21	20.6
		3.9	10		1.5	9.3	0.33	16	28.1

* Dogs had been rendered anemic by daily withdrawal of blood.

occurred in all 5 dogs. In dogs 3597 and 3575, the effective kidney function measured by any of the three parameters was diminished to less than 10% of its normal level.

DISCUSSION

The kidney function data reported in table 1 are in relatively close agreement with values assembled by Smith[12] for normal mature female dogs. The coefficients of variation ($100\sigma/m$) are higher than the expectation for a homogeneous group of dogs, but are probably not excessive for the present group in which great relative variations in age existed. Immaturity of tubular function in some of the younger dogs may have contributed to the variability to Tm_{PAH}.

Striking decreases in glomerular filtration rate, effective renal plasma flow, and Tm_{PAH} were observed in the dogs with severe responses to infection. In assessing the gravity of the kidney impairment in these animals, however, certain qualifications must be recognized.

The extreme reductions in glomerular filtration rate do not necessarily signify any extensive anatomical changes in the glomeruli themselves. Such factors as decreased arterial pressure, renal interstitial swelling, and dehydration may act to reduce glomerular filtration rate in the absence of specific glomerular damage. In addition, the possibility of diffusion of creatinine out of the damaged tubules must be considered. The effect that each of these factors may have in reducing glomerular effectiveness has not been determined, although the total effect of these and possibly other factors is a nearly complete abolishment of effective glomerular filtration.

Decreased arterial blood pressure, renal interstitial swelling, and dehydration may also act to reduce effective renal plasma flow. In addition, the extraction ratio of PAH may be considerably reduced in the presence of nephritis.[13] Extraction ratios were not determined on any of the dogs in this series. The values obtained for effec-

12. Smith, H. W., 1951, The Kidney, Structure and Function in Health and Disease, New York, Oxford Univ. Press.

tive renal plasma flow are therefore acceptable only as rough approximations in those animals with severe renal impairment. Alterations in the extraction ratio would not be expected in those dogs with little or no loss of renal function. The relative decreases in glomerular filtration rate and effective renal plasma flow were of the same magnitude in most instances. The filtration fraction was therefore relatively constant.

The tubular maximal excretory capacity for PAH (Tm_{PAH}) is less likely to be influenced by extrinsic variables than either glomerular filtration rate or effective renal plasma flow. The Tm_{PAH} is generally accepted as an index of the quantity of functional tubular excretory tissue and is regarded as quite independent of the amount of plasma perfusing the tubules, providing that the quantity of PAH presented to the tubules is sufficient to maintain the maximal rate of tubular excretion.[12]

The marked reduction in the Tm_{PAH} in those dogs classified as having severe infections is consistent with the anatomical changes observed in the kidney tubules by other investigators.[3,13] An attempt will be made to correlate the functional data with the specific lesions observed when histological studies now in progress have been completed.

SUMMARY

Renal function in immature dogs infected with *Leptospira icterohemorrhagiae*, CF-1, was studied by clearance methods. Exogenous creatinine was employed to estimate glomerular filtration rate and para-aminohippuric acid was utilized to measure renal plasma flow and Tm_{PAH}. In severe infections the glomerular filtration rate and renal plasma flow were reduced to as little as 10% of normal and Tm_{PAH} to 5% of normal. In moderate infections transient functional impairment occurred in those dogs having slight increases in blood urea nitrogen concentration. No significant change in renal function was found in dogs with subclinical or mild infections.

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13. Bloom, F. 1941, Cornell Vet. **31**: 266–288.

DETECTION OF COMPLEMENT FIXING ANTIBODIES AGAINST FOOT-AND-MOUTH DISEASE IN CATTLE SERUM

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The complement fixation test has become of considerable value in the early diagnosis of foot-and-mouth disease (F and M) in cattle and in typing the virus from this disease. However, this method is less useful in later stages of the disease when infected tissue can no longer be obtained for antigen. Furthermore, it is difficult to detect complement fixing antibodies in serums from convalescent cattle when using conventional procedures. Therefore, many attempts have been made to improve the sensivitity of the test. These modifications of the complement fixation test have included the use of unheated serum (Fredericks, 1949) or performance of the entire test at higher salt concentration (Serra and Guarini, 1951). None of these have proved to be successful.

An indirect method for demonstrating antibodies in late convalescent F and M serums has been suggested by Rice and Brooksby (1953). The indirect complement fixation test had earlier been applied by Rice (1948) in detecting antibodies in anti-pullorum serums, by Wolfe (1949) in anti-Newcastle disease serums, by Karrer et al (1950) and Hilleman et al (1951) in anti-ornithosis-psittacosis, and by Downie et al (1950) in anti-variola serums from chickens. Rice and Boulanger (1953) have also used it to demonstrate antibodies against vesicular viruses in cattle, and Rice and McKercher (1954) in studies of vesicular stomatitis in horses, cattle and swine. The difficulty with the indirect test is its relative complexity, and it is time consuming.

A simple modification of the F and M complement fixation test was described by Nordberg and Schjerning-Thiesen (1953) in a preliminary report. These workers were able to demonstrate antibodies against F and M in the serums of three hyperimmunized and seven convalescent cattle when a conventional test was employed but with temporary hypertonization of the serum component alone. The present report describes studies on a larger series of convalescent and vaccinated cows and presents the procedure in greater detail.

MATERIAL AND METHODS

A total of 56 convalescent serums were studied. The cattle were infected with different virus types, which included 40 of type 0, 10 of type A_5, 2 of type A, and 4 of type C. Forty-nine serums were obtained directly from Danish cattle and 7 serums were secured from the Laboratoire de Diagnostic et de Recherches de Veterinaires de l'Etat, Bruxelles. Twelve serums were obtained from vaccinated cows ("vaccine-immune" serums) on a farm in Denmark, where the stock had been vaccinated with a bivalent vaccine (A_5+C) of Danish manufacture.

As controls, 73 normal serums were obtained from healthy cows in areas of Sweden where F and M disease has never been demonstrated and consequently where the cattle had never been vaccinated against F and M.

The F and M antigens consisted only of heat-

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inactivated bovine apthous lymph (collected from 30 to 40 animals), in a 25% solution in 1/15 *M* phosphate buffer. Apthous epithelium used as antigen gave nonspecific reactions. The complement was obtained from guinea pigs and stored at 4 C diluted with Wittes (1940) fluid (2 g boric acid+5 g potassium sulphate+50 g aq. dest.) to 50%. Complement was always titrated before use and used in 2, 4, 6, 8 units required for 50% hemolysis. Rabbit hemolytic amboceptor was stored with merthiolate (1:10,000) and used in a quantity of 4 units. Sheep erythrocytes, washed 4 to 5 times in a sodium chloride solution, were used as a 2% suspension.

The technique of complement fixation was that used routinely for F and M virus typing at the State Veterinary Institute for Virus Research, Denmark. Fundamentally this technique is similar to that described by Brooksby (1952) for routine typing of F and M virus at the Research Institute (Animal Virus Diseases), Pirbright, England. In our investigations the modification consisted of inactivation of the serums by means of a temporary hypertonization, instead of by heating at 56 C for 30 minutes. The hypertonization was carried out by adding a definite amount (3 ml) of a 2.15% sodium chloride solution to an equal amount (3 ml) of serum. The mixture was then left at room temperature (20 to 24 C) for 2 to 4 hours. To the hypertonized serum (6 ml) was added an equal amount (6 ml) of a 0.2% sodium chloride solution, so that the final serum dilution (12 ml) again became isotonic. Further dilutions ($\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, etc.) were then made with a 0.85% sodium chloride solution. The experiments were performed within half an hour after restoration. Incubation consisted of immersion of the test in a 37 C water bath for 60 minutes, followed by addition of the hemolytic system and incubation for an additional 45 minutes. Following this the tests were placed at room temperature and read after 24 hours.

All of the serums were tested against antigens of type A, A_5, O (Lindholm O) and C viruses. In each experiment control tests were carried out in order to avoid using anticomplementary serums and antigen and also to control the specificity of the antigen. The hypertonized serums were always tested in parallel with the corresponding serums inactivated by heating alone. Only complete (++++) or almost complete (+++) fixation was considered as positive.

RESULTS

The results of the complement fixation test with acute and convalescent serums are given in table 1. It will be seen that specific complement fixation for the homologous type is demonstrable with serums which have undergone temporary hypertonization. In contrast, only in one case could a partial fixation be obtained with the heated serums. A breakdown of the exact titers obtained is shown in table 2. It will be seen that the majority of the type O serums reacted at 1:32, whereas with types C, A and A_5 the titers were somewhat lower.

The results obtained with the "vaccine-immune" serums are shown in table 3. From this it can be seen that the reactions here were nonspecific and there were more cross reactions than

TABLE 1.—*A comparison of the effect of hypertonization or heating (56 C for 30 minutes) on the sensitivity of the foot-and-mouth complement fixation test with acute and convalescent serums.*

Serum			Reaction							
			Serum hypertonized				Serum heated			
Number of animals	Type of virus	Days after infection	Antigen				Antigen			
			A	A_5	O	C	A	A_5	O	C
4	O	7	−	−	+	−	−	−	−	−
4	O	12	−	−	+	−	−	−	−	−
4	C	12	−	−	−	±	−	−	−	−
2	A	12	±	−	−	−	−	−	−	−
2	O	14	−	−	+	−	−	−	±	−
3	A_5	14	−	+	−	−	−	−	−	−
12	O	18	−	−	+	−	−	−	−	−
7	A_5	18	−	+	−	−	−	−	−	−
6	O	28	−	−	+	−	−	−	−	−
6	O	40	−	−	+	−	−	−	−	−
6	O	62	−	−	±	−	−	−	−	−

−, total hemolysis; ±, partial fixation; +, fixation.

TABLE 2.—*Distribution of the complement fixing titer of hypertonized cattle serums.*

Serum			Antigen			
Number	Type of virus	Dilution	A	A_5	O	C
40	O	1:4	—	—	5/40	—
		1:8	—	—	3/40	—
		1:16	—	—	32/40	—
4	C	1:4	—	—	—	2/4
		1:8	—	—	—	2/4
		1:16	—	—	—	0/4
2	A	1:4	1/2	—	—	—
		1:8	1/2	—	—	—
		1:16	0/2	—	—	—
10	A_5	1:8	—	4/10	—	—
		1:16	—	4/10	—	—
		1:32	—	2/10	—	—

Numerator = number positive; denominator = number tested.

TABLE 3.—*A comparison of the effect of hypertonization or heating (56 C for 30 minutes) on the sensitivity of "vaccine-immune" serums.*

Number of "vaccine-immune" serums (type A_4+C) bled 96 days after vaccination	Reaction							
	Serum hypertonized				Serum heated			
	Antigen				Antigen			
	A	A_4	O	C	A	A_4	O	C
1	—	1/4	—	1/4	—	—	—	—
2	—	1/4	1/4	1/4	—	—	—	—
3	—	—	—	1/4	—	—	—	—
4	—	1/8	1/8	1/8	—	—	—	—
5	—	1/16	1/8	1/4	—	—	—	—
6	—	—	—	1/8	—	—	—	—
7	—	—	1/4	1/4	—	—	—	—
8	—	—	—	1/4	—	—	—	—
9	—	1/8	1/4	—	—	—	—	—
10	—	1/8	—	1/8	—	—	—	—
11	—	1/4	1/4	1/4	—	—	—	—
12	—	—	—	1/4	—	—	—	—

—, total hemolysis. 1/4, 1/8, 1/16 = serum dilution with complete (++++) or partial fixation.

in the case of convalescent serums. No fixation occurred with heated serums.

In a control investigation using the normal serums from 73 healthy Swedish cattle no fixation was obtained with any of the antigens, either after a temporary hypertonization or after heating at 56 C for 30 minutes.

DISCUSSION

The findings seem to be in accord with those reported earlier by the authors (1953). Acute and convalescent serums tested 7 to 40 days after the onset of disease contained type-specific antibody, whereas after 62 days the level of fixation appears to drop. The "vaccine-immune" serums, obtained 96 days after vaccination, showed a weak and non-type-specific fixation. From these studies it appears that the complement-fixing antibodies in convalescent cattle serums progressively diminish after about 60 days until they finally disappear. Additional studies are required to determine whether this drop in antibody is associated with a decreased immunity in the animal.

Hypertonization of all the reacting components in the complement fixation test has been used by Milgrom and Bekierkunst (1950) with some success in the diagnosis of syphilis. They found that serums which failed to react with the components when in isotony gave positive results after hypertonization. These authors also tried temporary hypertonization of the serum component only, but without any effect.

These studies, as well as our own, suggest that a hypertonization of all the reacting components in the complement fixation or, a temporary hypertonization of the serum component, may in certain instances give results which are unobtainable with the conventional method.

The experiments with heated serums indicate that the complement fixing F and M antibodies are especially thermolabile and that they may be destroyed at temperatures which inactivate the serum's own complement. It is also possible that through the hypertonization of the serums the complement fixing F and M antibodies are subjected to some sort of disruptive effect which releases them from an inhibitor blocking their fixation to the homologous antigen.

SUMMARY

It has been found possible to demonstrate antibodies against foot-and-mouth disease more readily in cattle serum if the serum component in complement fixation is inactivated by means of a temporary hypertonization instead of by heating at 56 C for 30 minutes.

REFERENCES

Brooksby, J. B. 1952, The technique of complement fixation in foot-and-mouth disease research. A. R. C. Report ser. no. 12. London, Her Majesty's Stationary Office.

Downie, A. W. and Macdonald, A. 1950, A study of the pox viruses by complement fixation and inhibition of complement fixation methods. J. Path. & Bact. **62**: 389–401.

Fredericks, H. H. J. 1949, Het aantonen van complement-bindende stoffen ter bepaling van het smetstofftype in bloedserum van immune runderen, Tijschr. Diergeneesk. **74:** 839–848.

Hilleman, M. R., Haig, D. A. and Helmold, R. J. 1951, The indirect complement fixation, haemagglutination and conglutinating complement absorption test for viruses of the psittacosis-lymphogranuloma venerum group. J. Immunol. **66:** 115–130.

Karrer, H., Meyer, K. F. and Eddie, B. 1950, The complement fixation inhibition test and its application to the diagnosis of ornithosis in chickens and in ducks. J. Infect. Dis. **87:** 13–23.

Milgrom, F. and Bekierkunst, A. 1950, La reaction de fixation de l'alexine aux concentrations hypertoniques de NaCl. (Nouvelle methode de serodiagnostic de la syphilis). Rev. Immunol. **14:** 137–158.

Nordberg, B. K. and Schjerning-Thiesen, K. 1953, The presence of foot-and-mouth disease antibodies in hyperimmune serum and convalescent serum demonstrated by the complement fixation method. Nord. vet. med. **5:** 289–295.

Rice, C. E. 1948, Studies in Pullorum disease. XII. Technical of the indirect complement fixation test for activity with Salmonella pullorum antigens. Canad. J. Comp. Med. Vet. Sc. **12:** 130–136.

Rice, C. E. and Brooksby, J. B. 1953, Studies of the complement-fixation reaction in virus systems. V: In foot and mouth disease, using direct and indirect methods. J. Immunol. **71:** 300–310.

Rice, C. E. and Boulanger, P. 1953, The use of direct and indirect complement-fixation tests for the demonstration of antibodies for vesicular viruses in cattle. Proc. Am. Vet. Med. Assoc. 169–174.

Rice, C. E. and McKercher, P. D. 1954, Studies of the complement fixation in virus systems. VI: In vesicular stomatitis in horses, cattle and swine. J. Immunol. **73:** 309–317.

Serra, A. and Guarini, G. 1951, Sulla possibilita di dimonstrare la presenza di anticorpi devianti nel siero iperimmune antiaftoso. Clin. Vet. **76:** 321–329.

Witte, J. 1940, Ueber die Konservierung des Komplements durch Elektrolyte. Tierärztl. Wochsch. **25:** 293–294.

Wolfe, D. M., Kornfeld, L. and Markham, F. S. 1949, Simplified indirect complement-fixation test applied to Newcastle disease immune avian serum. Proc. Soc. Exper. Biol. & Med. **70:** 490–494.

EFFECTS OF ANIMAL VIRUSES ON HOST CELL METABOLISM

I. EFFECTS OF ENCEPHALOMYOCARDITIS VIRUS ON NUCLEIC ACIDS AND PHOSPHORUS FRACTIONS OF EHRLICH ASCITES TUMOR CELLS

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The comparative ease with which "large" amounts of bacterial viruses may be prepared, together with the facility with which the virus-host reaction can be handled, have made this biological system the most frequent choice of the scientist in studying the biochemical relations between host and virus. As a consequence of this work, many important aspects of the chemical and metabolic changes incident upon phage infection have been elucidated.[1] Unfortunately, the situation with respect to animal viruses is more complex. The small yield of animal virus per gram of infected tissue makes very difficult the isolation of quantities of animal virus sufficient for analysis. In addition, the complex interrelationships between the infected cells and the rest of the animal host present a complicating factor which is almost impossible to overcome. The use of tissue culture of animal cells could help to solve the latter problem, but obtaining enough tissue culture cells is just now becoming possible. The use of ascites tumor cells as host cells for virus infection in vivo is a compromise solution.

The present work reports the results of some in vivo studies with Ehrlich ascites tumor infected with encephalomyocarditis (EMC) virus. It will be shown that there were observable no changes in the purine and pyrimidine makeup of cellular nucleic acids incident upon infection, nor was there any change in the concentration of cellular phosphorus compounds. However, marked changes in what appears to be turnover of the cellular phosphorus fractions did occur.

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1. Evans, E. A. 1952, Biochemical Studies of Bacterial Viruses, Chicago, University of Chicago Press.

METHODS

Mice and tumor.—Female Swiss mice of about 25 g weight were used throughout. Two strains of Ehrlich ascites tumor were used.* One was Hauschka and Klein's stock Ehrlich ascites tumor of an essentially tetraploid character. When this tumor became bloody and too virulent, we changed to the hyperdiploid (Lettre) strain. Experiments of the types described below were done with both tumors with similar results.

Virus. Encephalomyocarditis.[2] *American Type Culture Collection M.P. 78.*—Virus titres are expressed as Reed & Muench LD_{50} end points after intracerebral inoculation in mice of 0.03 ml of appropriate dilutions.† The virus adapted readily to growth in Ehrlich ascites tumor. That growth did indeed take place was established by serial passage of 0.1 ml of infected ascitic fluid, titering 10^{-6}, into mice with 7-day-old Ehrlich ascites transplants. After an additional 2 days the ascitic cell suspension, about 7 ml, was harvested. Four such passages, involving a total volume dilution of 10^{-7}, yielded a fluid that still titered about 10^{-6}. Within a few hours after inoculation of virus the amount detectable in the ascitic fluid was greatly reduced. It subsequently

* Both strains of tumor were obtained from the Institute for Cancer Research, Philadelphia, Pennsylvania, through the courtesy of Dr. Theodore Hauschka.

† We are indebted to Dr. Karl Habel and his co-workers of this Institute for the early assays.

2. Helliwig, F. C., & Schmidt, E. C. H. 1945, Science, 102: 31–33.

rose again so that titers of about 10^{-6} or 10^{-4} were generally reached in the ascitic fluid after 24 hours of infection, with levels of 10^{-6} generally and 10^{-4} occasionally being reached after 48 hours. Marked oncolysis was frequently noted after 3 to 4 days of infection.

Phosphorus (P^{31}).—Total phosphorus determinations were done on H_2SO_4-$CuSO_4$-H_2O_2 digests, inorganic phosphate on trichloracetic acid (TCA) extracts, by the method of Gomoryi.[3]

Radioactive phosphorus (P^{32}) was determined as the insoluble ammonium phosphomolybdate salt, in the presence of carrier phosphate.[4]

DNA.—Desoxyribonucleic acid was prepared by the method of Kay et al.[5] Assays for DNA in tissues and extracts were done on hot trichloracetic acid extracts[6] by the dephenylamine method of Dische.[7]

RNA.—Ribosenucleic acid was prepared by the method of Kay et al.[8] Assays for RNA in tissues and extracts were done on hot TCA extracts[6] by the phloroglucinol method of von Euler.[9]

Purine and pyrimidine determinations were made on concentrated formic acid hydrolysates of the nucleic acids by the method of Chargaff et al.[10]

Fractionation of tissues into biochemical components.—Two methods were employed for fractionation of the cells. (1) In the first, used for the radioisotope work and for purine and pyrimidine determinations, purified RNA and DNA were prepared by the methods of Kay et al.[5,8] DNA, so prepared, had 1 to 2% RNA, and RNA had less than 5% DNA. (2) The second method was used both for radioactivity studies and for quantitative determinations of the amount of each fraction in the cell, with some differences for the two purposes as indicated. The cells were removed from the animal, chilled and centrifuged out from the ascitic fluid. They were washed three times with ice-cold 0.85% saline, suspended in saline and aliquots removed for dry weight and specific activity determinations. The remainder of the cells were ground with cold 10% TCA in a glass homogenizer and washed with additional 10% TCA. The two TCA extracts were combined. The residue was washed with alcohol, then refluxed for 30 minutes with 3:1 alcohol-ether, and finally washed with ether. The combined organic solvents were considered to be the phospholipid fraction. The residue was then treated in one or the other of the two ways indicated in the next two paragraphs.

For radioactivity studies the cellular residue was extracted twice with 1*M* NaCl in 0.05*M* barbiturate at pH 7.4[11] in a boiling water bath for 30 minutes each time, and the extracted nucleic acids were precipitated with acid-alcohol. The crude nucleic acids were treated with 0.3 *N* KOH at 37 C overnight, and any insoluble residue centrifuged off. KOH was precipitated with $HClO_4$; the DNA was precipitated by acid alcohol, redissolved in NaOH and reprecipitated by HCl. The RNA hydrolysate was adjusted to pH 3.5 and fast-moving ionic phosphates separated by short time ionography.[12] The hydrolyzed RNA was eluted with HCl and analyzed.

For quantitative analysis for cellular components, the residue, after extraction of acid soluble components and phospholipids, was analyzed for nucleic acids by the colorimetric methods indicated above.

RESULTS

I. Effect on nucleic acid composition

While the amount of virus formed would be too small to affect directly the purine and pyrimidine composition of the nucleic acids of the virus-host system, it was thought possible that in some other way virus infection might lead to some changes in cellular nucleic acid composition. Accordingly purified RNA and DNA were prepared from 24 and 48 hour infected tumor cells initially infected intraperitoneally with 0.1 ml of EMC virus of 10^{-5} titer 7 days after tumor inoculation, and analyzed for

3. Gomoryi, G. A. 1942, J. Lab. & Clin. Med. **27**: 957–960.
4. Treadwell, F. P. and Hall, W. T. 1942, Analytical Chemistry, vol. II, New York, John Wiley & Sons, Inc., p. 373.
5. Kay, E. R. M., Summons, M. S. and Dounce, A. L. 1952, J. Am. Chem. Soc. **74**: 1724–1726.
6. Schneider, W. C. 1945, J. Biol. Chem. **161**: 293–303.
7. Dische, Z. 1930, Mikrochemie, **8**: 4–32.
8. Kay, E. R. M. and Dounce, A. L. 1953, J. Am. Chem. Soc. **75**: 4041–4044.
9. von Euler, H. and Hahn, L. 1946, Svensk. Kem. Tid. **58**: 251–265.
10. Chargaff, E., Lipshitz, R., Green, C. and Hodes, M. E. 1951, J. Biol. Chem. **192**: 223–230.
11. Levy, H. B., Skutch, E. T. and Schade, A. L. 1949, Arch. Biochem. **24**: 206–215.
12. Davidson, J. N. and Smellie, R. M. S. 1952, Biochem. J. **52**: 594–599.

TABLE 1.—*Effect of EMC infection on purine and pyrimidine composition of the nucleic acids of Ehrlich ascites tumor cells.*

	Mole percent composition			
	RNA		DNA	
	Virus	Control	Virus	Control
Adenine	19.1	18.7	30.8	30.7
Guanine	35.2	37.0	18.5	19.4
Cytosine	28.1	28.4	19.5	20.6
Thymine			31.2	29.2
Uracil	17.5	15.9		

TABLE 2.—*Analyses of infected and uninfected Ehrlich ascites tumor. All values are expressed as percent of the weight of the dry cells.*

	24-hour		48-hour	
Percent P in:	Control	Infected	Control	Infected
TCA Extracts	0.56	0.55	0.53	0.56
Organic Extracts (p-lipid)	0.28	0.26	0.28	0.26
Percent DNA	10.8	11.9	10.2	10.4
Percent RNA	12.6	13.1	12.6	12.3

purine and pyrimidine composition. In other experiments RNA nucleotides and DNA were prepared by hydrolysis of the washed cells in 0.3*N* KOH at 37 C overnight, followed by removal of KOH with $HClO_4$, and precipitation of DNA with acid-alcohol. In the later experiments DNA, after reprecipitation, was analyzed as before, but the RNA nucleotides were separated and determined by ionography by the method of Davidson and Smellie.[12]

In repeated experiments, no consistent differences were found between the RNA and DNA composition as a result of infection. Some small differences were occasionally found, but these were probably within experimental error, and were not reproducible.

Typical analyses are given in table 1.

II. Effect of EMC on concentration of phosphorus-containing compounds

Animals with 7-day-old Ehrlich ascites tumor cells were inoculated intraperitoneally with 0.1 ml of EMC virus of 10^{-3} titer, the cells were removed from 10 sacrificed animals after 24 and after 48 hours of infection. Control animals with uninfected cells were treated in the same way. The cells were analyzed for acid soluble P compounds, phospholipids, DNA and RNA, according to the scheme given in *Methods*. Results of a typical experiment are given in table 2.

It can be seen that no differences could be found between infected and noninfected cells. Similar results were obtained in 3 other experiments, although the absolute amounts of RNA varied between 7 and 13% from experiment to experiment.

III. Effect on turnover of cellular phosphorus fractions

In using radioactive tracers to study metabolic activity or turnover rates of metabolite, the extent of incorporation of a radioisotope is often used. Analyses of the results obtained by this method may be complicated by the fact that synthesis of new metabolite from the radioactive medium can result in large changes in the specific activity of the metabolite independent of any turnover. It was felt that this effect could be minimized by measuring the decrease in radioactivity from tumor cells labelled prior to infection. A detailed description of an experiment will present the justification of this approach.

Each of 60 mice bearing 8-day-old Ehrlich ascites tumor was inoculated intraperitoneally with 0.1 ml P^{32} (AEC catalogue no. P-1, diluted with lightly buffered 0.85% NaCl) containing 9 uc P^{32}. After allowing 24 hours for the cell to incorporate P^{32}, 20 mice were sacrificed for the determination of "zero time" values. The cells were removed quantitatively, fractionated as described under methods, and the specific activity, as well as the total phosphorus, of the several fractions, determined. (At this same time each of the remaining 40

TABLE 3.—*Effect of synthesis of new cell substance on specific activity of cellular phosphorus.*

Group	(1) Total P γ/2 ml suspension†	(2) New P made	(3) New c.p.m. added‡	(4) SA of P of (1)	Uncorrected percent D*	(5) Total c.p.m. present (1)×(4)	Total c.p.m. corrected for new counts (5)−(3)	New SA 6 ÷total P at zero time	Corrected percent D*
Zero time	1376			105		144,480		105	
Virus	1484	108	11,988	72	31	106,848	94,860	68.9	34
Control	1592	216	23,976	83	21	132,136	108,160	78.6	25

SA = c.p.m./γP.
* Percent D = Percent decrease in SA from zero time. Here used as an indication of the extent of turnover.
† Comparable aliquots of the total cells obtained from the group of animals.
‡ (2) ×specific activity of zero time ascitic fluid.

animals received, intraperitoneally, 3.6 mg of P in the form of a neutral mixture of Na_2HPO_4 and NaH_2PO_4. The purpose and effect of this injection is discussed in the next section). Twenty of the remaining 40 animals received an intraperitoneal inoculation of EMC with a titer of 10^{-8}. After an additional 24 hours the 20 infected and 20 control animals were sacrificed, and the tumor cells analyzed as before. From the data so obtained the amounts of newly synthesized P compounds were calculated, and on the assumption that their specific radioactivity (SA) was the same as that of the "zero time" ascitic fluid, the effect of the newly synthesized material on the SA of the fraction was determined. Table 3 shows the calculations involved, using total cell P as the example. It can be seen from this table that by use of the "loss of radioactivity" technique, the effects of small amounts of synthesis can be neglected.

In table 4 is presented a summary of the data obtained for the remainder of the metabolic fractions in three experiments of this type. (No corrections have been made for new synthesis.)

In all cellular fractions the SA of the fraction from the virus infected cells decreased more that did the corresponding fraction from the uninfected cells. The difference between the SA of the P-lipid fractions and of the TCA soluble fractions was small, but the constancy suggests that it was real. In view of the changing SA of the ascitic fluid with time, one cannot quantitatively relate the drop in SA from zero time (percent D) to phosphorus turnover, but should regard them as qualitative indications of the extent of such turnover.

In two other experiments using this

TABLE 4.—*Effect of EMC infection on decrease in SA of cellular phosphorus fractions.*

Fraction	Cells SA*	Cells Percent D†	TCA solution SA*	TCA solution Percent D†	P-lipid SA*	P-lipid Percent D†	DNA SA*	DNA Percent D†	RNA SA*	RNA Percent D†	SA of IP of ascitic fluid
I											
Zero time	105		206		80		80		76		111
Virus	72	31	159	23	58	27	50	37	54	29	54
Control	83	21	171	17	61	24	65	19	69	9	58
II											
Zero time			160		104		97		90		41
Virus			112	30	78	25	71	27	73	19	21
Control			120	25	83	20	85	13	77	14	20
III											
Zero time			184		73		80		83		58
Virus			124	33	57	22	62	22	63	24	25
Control			132	29	62	15	70	12	73	11	27

* SA = specific activity as c.p.m./γP.
† Percent D = percent decrease in specific activity from zero time. Here used as an indication of the extent of turnover.

technique of loss of radioactivity from cells labelled prior to infection, DNA and RNA were isolated as relatively undegraded compounds (see methods), and the SA of their phosphorus determined. As in the preceding section virus infection effected a 30 to 100% increase in percent D of RNA and DNA, as compared to that of the nucleic acids of uninfected cells.

An observation was made incidental to the experiments just described which may be worth reporting here. In the description of the experimental procedure for determining turnover rates, it was mentioned parenthetically that at the time of virus injection P^{31} was also injected intraperitoneally. The purpose of the P^{31} addition was to reduce the specific activity of the ascitic fluid so that any newly incorporated metabolite would have a low specific activity and so would not contribute significantly to the total radioactivity of the isolated metabolite. The amount of injected P^{31} was approximately ten times that normally present in the ascitic fluid, but, 24 hours after injection of the P^{31}, the specific activity of the inorganic phosphate of the fluid was about one-half that of the value immediately prior to injection, and the inorganic phosphate content was no higher than prior to injection. In order to find some explanation of these findings, 40 animals with 8-day Ehrlich ascites tumor were injected with 10 uc of P^{32}. Five animals were sacrificed immediately, after 5 hours, and after 24 hours, and the cells removed. At this time the remaining animals each received 3.6 mg of phosphorus as before, and 5 animals were sacrificed immediately, after 1 hour, 7 hours, 24 hours and 48 hours. The inorganic phosphate content and the specific activity of the inorganic phosphate were determined on each group of ascitic fluids. The results are represented on semilogarithmic scale in figure 1.

It will be seen that the specific activity (SA) drops rapidly after injection of P^{32}. Upon injection of P^{31}, the inorganic phosphate content of the fluid rises sharply, with consequent drop in specific activity. Thereafter, the inorganic phosphate drops rapidly back to normal and the SA rises. A likely explanation for these findings is that there is a rapid interchange of inorganic phosphate (IP) between the ascitic fluid and that of the rest of the animal. The addition of P^{31} results in a transient rise in IP in the ascitic fluid, but the reserve "capacity" of the body rapidly takes up this excess ascitic fluid IP, at the same time that the higher specific activity IP in the body brings up the specific activity of the fluid.

DISCUSSION

That virus infection has no effect on the concentration of the several phosphorus fractions nor on the purine and pyrimidine makeup of the nucleic acids is not surprising. Previous reports in the literature are in agreement with these findings.[13,14] The amount of virus produced by each gram of cells certainly does not exceed a few micrograms and is probably a good deal less, so that one would not find any change in composition directly attributable to the composition of the virus present. The extent of any indirect effects of virus multiplication would be expected to depend upon the fraction of cells that were infected and the amount of virus produced by each cell. Neither of these quantities is readily determinable under the conditions of this experiment, but comparison with the yield of virus per unit weight

13. Zahler, S. A. and Moulder, J. W. 1953, J. Infect. Dis. **93**: 150–158.
14. Hultin, T., Gustafsson, K. and Wessel, G. 1954, Exper. Cell. Res. **7**: 345–350.

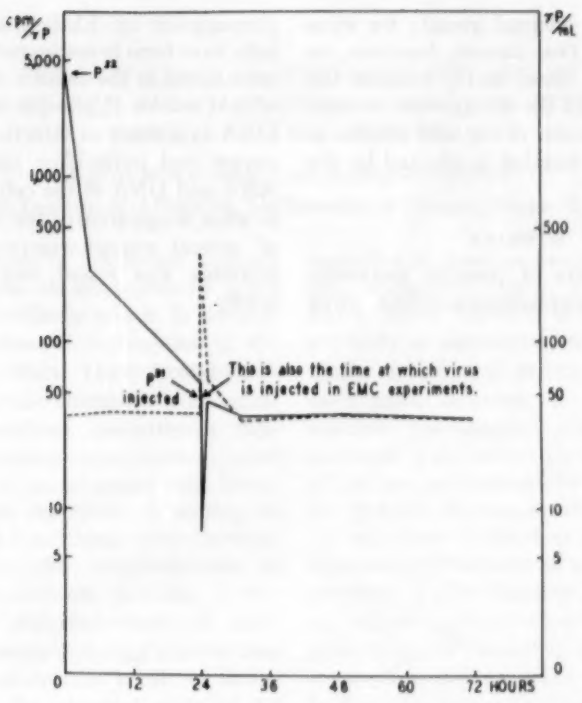

FIG. 1.—Changes in SA of ascitic fluid following intraperitoneal injection of P^{32}

of tissue in tissue culture suggests that the yield of virus from the Ehrlich ascites tumor is relatively low.

It is of some interest, therefore, that a consistent increase in what is apparently the turnover rate of several metabolic components is occasioned by EMC propagation in ascites cells. Twenty-four hours after infection RNA phosphorus and DNA phosphorus turnover is markedly increased. Zahler and Moulder,[13] using a very different approach, reached similar conclusions about the effect of feline pneumonitis virus on the chick embryo yolk sac. Hultin, Gustaafsson and Wessel[14] found that influenza virus caused an increased uptake of P^{32} by chorioallantoic membranes, which effect they attribute to a change in cellular permeability caused by the virus. It is difficult to see how changes in permeability could account for the results reported here, but this must be studied further. The fact that the turnover of the acid soluble P of virus infected cells does not show as great an increase under the conditions of this experiment as that of the RNA-P and DNA-P requires some consideration. It is likely, though not certain, that the radioactive phosphorus enters the cell as inorganic phosphate. If the sole effect of the virus is to increase permeability of the cell, then one would expect that the ratio of the specific activity of the virus fraction to that of the control fraction would be essentially constant for all the different fractions. Since this is not the case, it would appear that the process of conversion of inorganic phosphate to the various metabolites of the acid soluble

group is not affected greatly by virus propagation. One cannot, however, on the basis of these data, exclude the possibility that the metabolism at some minor constituent of the acid soluble or phospholipid fraction is affected by the virus.

SUMMARY

Some aspects of possible metabolic effects of encephalomyocarditis virus propagation on Ehrlich ascites tumor cells have been investigated. No changes were found in the cellular concentration of acid soluble P, phospholipid, RNA or DNA as a result of infection, nor in the purine and pyrimidine composition of RNA and DNA of the cell. An increase in what is apparently the turnover rate of several phosphorus-containing metabolites was found, and is discussed briefly.

THE BLOCKING OF LABELED ANTIBODY UPTAKE AS A MEASURE OF THE RELATIVE COMBINING CAPACITY* OF ANTI-RED CELL SERUMS

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Most methods of antiserum analysis depend on the measurement of some activity of antibody which is secondary to the primary combination of antigen and antibody. These second stage reactions include precipitation, agglutination, hemolysis, complement fixation, bacteriolysis, opsonization, viral neutralization, anaphylaxis and histamine-like skin reactions. According to the unitarian hypothesis of antibodies,[1] differences in the manifestations of antibody activity are not due to the existence of different types of antibodies, i.e., precipitins, agglutinins, reagins, etc., but are the result of differences in (a) the physical state of the antigen, (b) the specificity and reactivity of the antibody, and (c) the circumstances of the antigen-antibody union. While differences in the physical properties of antibodies are known to exist, there is no good evidence of an association in a monospecific serum between a given set of physicochemical properties and a particular manifestation of antibody activity.[2] A number of examples of possible exceptions to the last statement might be cited[3,4] in which some unusual activity of an antibody is associated with some particular fraction of serum globulin. In each case, however, it is not clear whether the unusual activity of the antibody is dependent on the specificity of the antibody or on the properties of the globulin fraction in which it resides.

This paper is the first of a series of reports which consist of a study of the problem of the diversity of hemolytic activity of anti-red cell antibodies. The present paper describes a method for measuring the capacity of the antibodies of a serum to combine with an insoluble antigen, the sheep red cell. Such a method is required to compare the combining capacity of the serum with its hemolytic activity.

The red cell system seemed at the outset to promise certain advantages to a study of diversity of antibody. The hemolytic activity of a serum can be

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* The term "combining capacity" is used here to indicate that its measurement is based on the first stage antigen-antibody reaction (i.e., the physical combination of antigen and antibody) rather than on a second stage reaction such as precipitation or hemolysis. For a fuller explanation, see *Discussion*.

1. Bronfenbrenner, J. 1948, Hypersensitivity and immunity in the light of the "Unitarian" hypothesis. J. Allergy, 19: 71–89.
2. Wilson, G. S. and Miles, A. A. 1946, Topley and Wilson's Principles of Bacteriology and Immunity, vol. I, Baltimore, The Williams and Wilkins Co., p. 252.
3. Hill, J. M., Haberman, S. and Guy, R. 1949, Further evidence for antibodies of third order: fractionation of agglutinins, blocking antibodies and cryptagglutinoids by physicochemical methods. Am. J. Clin. Path. 19: 134–140.
4. Kuhns, W. J. 1954, Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. IV. Differences between human precipitative and nonprecipitative skin sensitizing diphtheria antitoxin as shown by electrophoresis. J. Exper. Med. 99: 577–588.

measured with sensitivity and precision using the 50% hemolytic unit. Croft has demonstrated a lack of correlation between agglutinin nitrogen and hemolytic activity,[5] and Taliaferro and Talmage have found that hemolysin turned over in vivo at a rate nearly twice that previously reported for precipitating antibody.[6] Both these findings suggest that two different types of antibody may be present in anti-red cell serums.

A major difficulty was encountered in measuring the antibody content of the anti-red cell serums. For this type of study, the method used must be based on the primary combination of antigen and antibody (first stage reaction), since it is desired to relate antigen-antibody union to a particular manifestation of this union (hemolysis). A nitrogen analysis of absorbed antibody would be satisfactory were it not for certain technical problems which have been discussed in detail by Croft[5] and by Marucci.[7] In addition to the lack of sensitivity of the nitrogen determination (relative to hemolysin titration), this method involves a large and uncertain contribution of antigen (and nonantigen) nitrogen to the aggregate on which the nitrogen determination is made.

The use of a purified cellular antigen, such as the lipopolysaccharide isolated by Rapp,[8] would eliminate a number of problems associated with nitrogen measurement, but would also introduce another second stage reaction (precipitation), which complicates the interpretation of results. Agglutination titers, while sensitive, are not sufficiently precise and likewise involve a complicating second stage reaction (agglutination).

Butement's demonstration of the uptake of I^{131} trace-labeled antibody by *Proteus vulgaris* bacilli[9] suggested that a similar technique might be used to measure anti-red cell antibodies which would avoid the above mentioned limitations of nitrogen measurement and would depend solely on the primary combining capacity of the antibody. Marucci studied the labeling of normal serum globulins and antibody globulin with I^{131} [7] and concluded that the use of radioiodinated antibody for quantitative antibody analysis was not feasible because of the difficulty in determining the specific radioactivity of the antibody protein. Additional difficulty lies in the separation of weak antibody from proteins nonspecifically absorbed by the antigen.

The method reported here for measuring the combining capacity of an antiserum was made possible by the development of techniques for preparing highly specific labeled antibody solutions.[10] The basis of the method is the blocking of the uptake by red cells of an I^{131} trace-labeled antibody. The number of cells and the amount of labeled antibody are adjusted so that when little or no test serum is added, most of the labeled antibody is ab-

5. Croft, C. C. 1949, Studies on the quantitative determination of hemolytic antibody. Thesis, Johns Hopkins University.
6. Taliaferro, W. H. and Talmage, D. W. 1954, Two antibodies in the rabbit with different rates of metabolic decay. Fed. Proc. **13**: 514.
7. Marucci, A. A. 1954, A critical study of the use of radioiodinated antibody as a tool in antibody analysis. Thesis, Johns Hopkins University.
8. Rapp, H. J. 1953, Purification and immunochemical characterization of the heat stable, alcohol-soluble hemolytic antibody inhibitor of the sheep erythrocyte. Thesis, Johns Hopkins University.
9. Butement, F. D. S. 1948, Introduction of radioactive tracers into antisera. Nature, **162**: 731.
10. Talmage, D. W., Baker, H. R. and Akeson, W. 1954, The separation and analysis of labeled antibodies. J. Infect. Dis. **94**: 199–212.

sorbed. When the amount of the test serum added is increased to the point that most of the combining sites on the red cells are blocked by unlabeled antibody, the uptake of labeled antibody by the red cells decreases. The end point chosen for measuring combining capacity is that amount of serum producing 50% inhibition of uptake of labeled antibody. In this determination, the serums tested are not labeled with I^{131} but compete with or block a labeled antibody which has been prepared beforehand. This manipulation has two advantages: first, one labeled antibody preparation may be used to test a large number of serums, and second, the antibody to be titrated is not labeled and cannot be altered by the labeling procedure. As compared to the quantitative agglutinin nitrogen determination, the test is simpler to perform, avoids the uncertainty of the contribution of antigen nitrogen and measures the combining capacity of the serum under the more competitive conditions existing in antibody excess.

Although the test was developed for anti-red cell serums, a fact which simplifies standardization of antigen, it should be possible to adapt this procedure to any insoluble antigen.

MATERIALS AND METHODS

Rabbits were immunized by the intravenous injection of either a sheep red cell suspension or a suspension of heated sheep red cell stromata prepared according to Jaroslow and Taliaferro.[11] Before heating, stromata were counted in a hemocytometer chamber with a phase contrast microscope. They were heated at 103 C for 60 minutes. Serums from animals injected with heated stromata are referred to as anti-Forssman serums. Details of dosages and injection schedules are given in each experimental section. Rabbit antiserum to bovine serum albumin (anti-BSA) was prepared by two injections a month apart of 30 mg/kg BSA, Armour Laboratories lot #N12403.

To obtain serums for labeling, animals were bled by intracardiac puncture, usually 7 days after the last injection, and the globulins of the serum after precipitation at 50% saturation with $(NH_4)_2SO_4$ were trace-labeled with I^{131} by methods detailed by Talmage, Baker and Akeson.[10] Nitrogen analysis of serum globulin fractions was made by a method[12] similar to the Lowry modification of the Folin procedure.[13]

Radioactive samples were counted in Wassermann tubes in a well-type scintillation counter. For each experiment, the volume in all of the tubes was equalized with water to give comparable geometry for counting.

The nitrogen content of stromata-antibody aggregates was analyzed by Lanni et al's modification[14] of the direct-nesslerization procedure of Koch and McKeekin.[15]

Labeling of stromata with I^{131}.—To 1 mc I^{131} was added 0.1 ml of 0.01 *N* KI and 1 drop each of 0.5 *N* HCl and 0.1 *N* $NaNO_2$. To this light iodine-colored solution was added 2 ml of a suspension of heated stromata containing 1.6×10^{11} stromata in a 0.1 *M* carbonate buffer pH 10. The labeled stromata were dialyzed overnight against running tap water and for 3 days against several changes of 1:10,000 merthiolated 0.85% saline. The stromata were then centrifuged in an average field of $20,000 \times g$ for 30 minutes and resuspended in 10 ml of merthiolated saline. One ml of this suspension contained 6 million counts per minute (approximately 10 μc).

Hemolysin titration and red cell standardization.—Hemolysin titrations were performed and red cell suspensions were prepared and standardized colorimetrically according to the methods of

11. Jaroslow, B. and Taliaferro, W. H. 1956, The restoration of hemolysin forming capacity in x-radiated rabbits by tissue and yeast preparations. J. Infect. Dis. **98**: 75–81.
12. Freter, G. L. 1955, Studies of Forssman antibodies: The measurement of combining capacity and its relation to hemolytic activity. Thesis, The University of Chicago.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951, Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.
14. Lanni, F., Dillon, M. L. and Beard, J. W. 1950, Determination of small quantities of nitrogen in serological precipitates and other biological materials. Proc. Soc. Exper. Biol. & Med. **74**: 4–7.
15. Koch, F. C. and McMeekin, T. L., 1924, New direct Nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Am. Chem. Soc. **46**: 2066.

W. H. and L. G. Taliaferro.[16] One ml of 1% red cell suspension (approximately 1.6×10^8 cells) was estimated to contain 2.5 mg hemoglobin on the basis of dry weight. For convenience, quantities of red cells used are at times given on this basis, i.e., in micrograms red blood cell hemoglobin (RBC Hgb).

EXPERIMENTAL PROCEDURE AND RESULTS

Determination of hemolytic activity per unit weight of antibody

As a basis of comparison with the competitive absorption test to be described, it was desired to know the ratio of hemolytic units to weight units in a single high titer anti-Forssman serum.

The amount of antibody nitrogen and the amount of hemolytic activity absorbed from 1 ml of an anti-Forssman serum by 1 ml aliquots of I^{131}-labeled stromata suspension were determined according to the protocol outlined in table 1. The serum used for this experiment contained 38,000 hemolytic units per ml. The number of stromata used was adjusted to absorb only about one-half the hemolysin in order to decrease relatively the absorption of weak antibody. Control tubes were made (1) with normal rabbit serum to measure the nonspecific absorption of protein, and (2) with stromata alone to measure their relative specific radioactivity. Both the normal rabbit serum and the anti-Forssman serum were decomplemented beforehand by mixing for 30 minutes at 37 C with an unrelated immune precipitate (BSA-anti-BSA, 60 μg N/ml serum).

The stromata-serum mixtures (table 1) were agitated at room temperature for 20 minutes and centrifuged in an average field of $20{,}000 \times g$ for 30 minutes. The stromata were then washed three times at room temperature with 2 ml aliquots of a mixture of 9 parts of 0.15 *N* NaCl and 1 part 0.15*N* KI. The supernatants and washes were analyzed for hemolytic activity. Since the washes contained only trace amounts of hemolysin, the number of hemolytic units in the supernatant was subtracted from the number originally present in the serum to give the number of hemolytic units absorbed by the stromata.

The washed stromata were suspended in 2 ml of 0.04 *N* NaOH and 0.2 ml aliquots were removed from each tube for nitrogen analysis. One ml of a 1:100 dilution was taken from each tube for counting.

Tubes 6 and 7, which contained no serum, served to indicate the relative specific radioactivity of the stromata. In these two tubes, the average ratio of I^{131} counts in the aliquot to μg stromata nitrogen was 1620. The number of counts obtained in the aliquots of the first five tubes was in each case divided by 1620 to give the number of μg stromata nitrogen in that aliquot. The

16. Taliaferro, W. H. and Taliaferro, L. G. 1950, The dynamics of hemolysin formation in intact and splenectomized rabbits. J. Infect. Dis. **87**: 37–62.

TABLE 1.—*Ratio of hemolytic units to antibody N absorbed by heated stromata.*

Tube	Serum added	I^{131} counts in aliquot	Total N in aliquot	Counts per μg N	Stromata N in aliquot	Absorbed N in aliquot	Antibody N in aliquot	Total antibody N absorbed	Hemolytic units absorbed	Hemolytic units per μg antibody N
1	Anti-F	29,900	23.5*		18.1	5.4	5.1	51	14,000	274
2	Anti-F	29,300	24.0		17.8	6.2	5.9	59	16,000	271
3	Anti-F	29,800	24.0		18.1	5.9	5.6	57	14,000	250
4	NRS†	26,800	17.1		16.5	0.6				
5	NRS†	28,300	17.3		17.4	.0				
6	None	27,700	17.6	1570						
7	None	26,700	16.0	1670						

* Nitrogen is given in μg.
† NRS is normal rabbit serum.

nitrogen attributable to stromata was then subtracted from the total nitrogen to give the absorbed nitrogen.

Tubes 4 and 5 served to indicate the nonspecific absorption of protein from normal rabbit serum. The average of 0.3 μg nitrogen per aliquot was subtracted from the absorbed nitrogen in tubes 1, 2 and 3 to give the absorbed antibody nitrogen in the aliquot. This was multiplied by 10 to give the total antibody nitrogen absorbed. The average number of hemolytic units absorbed per μg nitrogen was 265. This ratio can not be used to determine the weight of antibody N per hemolytic unit because, as shown in the next section, part of the absorbed antibody is relatively nonhemolytic.

Absorption of labeled antibody and hemolytic activity from labeled globulin fractions

Serums were obtained from five rabbits 1 week after a 3-week course of thrice weekly intravenous injections of 1 ml/kg of a heated stromata suspension containing 1.6×10^9 stromata per ml. The globulin fractions of each of these five serums and of one normal rabbit's serum were labeled with I^{131}, and absorption studies were carried out using varying numbers of sheep red cells as follows: after standing for 30 minutes at room temperature, the cell-globulin mixtures were centrifuged for 10 minutes in an average field of $150 \times g$* and the sedimented cells washed three times at room temperature with 2 ml aliquots of a mixture of 10 parts 0.85% NaCl, 1 part 0.15 *N* KI and 1 part normal rabbit serum. The normal rabbit serum was heated at 56 C for 1 hour to inactivate complement and was absorbed with sheep red cells to remove natural antibody. The percent radioactivity absorbed was determined from counts of the supernatant, precipitate and all the washes. The percent hemolytic activity absorbed was determined by comparing the hemolytic titer of the supernatant with that of the unabsorbed labeled globulin.

TABLE 2.—*Absorption of I^{131} globulin from 5 immune serums by sheep red cells.*

Labeled globulin fraction of serum	Percent I^{131} absorbed by 600 μg RBC Hgb per hemolytic unit	Absorption by 6 μg RBC Hgb per hemolytic unit	
		Percent I^{131} absorbed	Percent hemolytic units absorbed
1	0.75	0.23	>97
2	0.99	0.19	>97
3	0.61	0.22	>98
4	1.29	0.27	>96
5	0.77	0.15	>97
Control	0.17	0.02	

It was determined from preliminary experiments that 6 units of cells in μg RBC Hgb per hemolytic unit would absorb most of the hemolytic activity. Table 2 gives the results obtained following absorption of labeled globulin from the 5 serums with 6 and 600 units of cells in μg RBC Hgb per hemolytic unit. With 6 μg of RBC Hgb the cells absorbed more than 96% of the hemolytic activity in each case but only one-third to one-fifth of the total radioactivity absorbable by 100 times that number of cells. This additional absorption of radioactivity by numbers of red cells greater than that required to absorb most of the hemolytic activity indicates the presence of antibody of low hemolytic activity. That this weakly hemolytic or nonhemolytic antibody was not produced by damage to hemolytic antibody in the labeling procedure is indicated by a comparison of the hemolytic titer in the original globulin and the labeled globulin (table 3). Since an uncertain loss of the globulin occurred in the labeling procedure, this comparison is made on the basis of the

* A greater centrifugal force will pack the red cells so that they cannot be easily broken up for washing.

TABLE 3.—*Hemolytic activity of labeled and unlabeled globulin from 5 immune serums.*

Serum	Hemolytic units per μg N in	
	Unlabeled globulin	Labeled globulin
1	2.32	2.19
2	1.85	2.10
3	3.49	3.00
4	1.72	1.83
5	2.32	2.36

ratio of hemolytic units to μg globulin nitrogen.

Separation of two labeled Forssman antibodies

In this section, methods are described for preparing two labeled antibody eluates, one containing the labeled antibody absorbable by 6 units of cells in μg RBC Hgb per hemolytic unit and the other using larger numbers of red cells. A comparison of these two eluates illustrates the reason for using the first eluate in the competitive absorption test described below.

Serum was taken from animals after 2 to 3 weeks of thrice weekly injections of heated stromata. Each injection consisted of 1 ml/kg of a heated stromata suspension containing 1.6×10^9 stromata/ml. Ten mg of the globulin fraction† were labeled with 5 mc I^{131}. After removal of nonprotein bound iodide and adjustment of pH to 7.0, the labeled antibody was absorbed on sheep red cells (6 μg RBC Hgb per hemolytic unit in the labeled globulin). The absorption was performed at 37 C for 30 minutes and was followed by 3 washes at 37 C with 5 ml aliquots of the saline-KI-serum mixture described in the previous section. Fifty to 70 percent of the labeled antibody was then eluted by a neutral exchange reaction[10] for 15 minutes at 45 C using whole anti-Forssman serum containing 5 times the number of hemolytic units present in the labeled globulin fraction or 5 hemolytic units for each 6 μg RBC Hgb used for absorption. Both the normal rabbit serum used in the washes and the antiserum used for the elution were heated before use at 56 C for 30 minutes to inactivate complement. The eluate obtained after centrifugation of red cells is referred to as the first anti-Forssman eluate. Eighty to ninety percent of the radioactivity of this eluate could be absorbed on excess red cells. Until used, it was kept undiluted at 4 C. Merthiolate was added to a final concentration of 1:10,000.

A labeled globulin solution from which the most avid antibody had been absorbed to prepare the first eluate was used to prepare the second eluate. To this globulin was added an amount of red cells containing 6 μg hemoglobin for each hemolytic unit present before the first absorption. This is the same number of red cells used in the first absorption. The cells were washed three times as before, except this time at room temperature. The antibody was then eluted by adding 4 ml of normal rabbit serum and incubating at 37 C for 15 minutes. Antiserum for an exchange reaction was not added in this case since it was desired to obtain only the least avid antibody in the eluate. Approximately 50% of the radioactivity in the eluate could be absorbed on excess red cells. This is referred to as the second anti-Forssman eluate.

Absorption of first and second anti-Forssman eluates by red cells

Unlabeled anti-Forssman serum was added to the second eluate and then the two eluates were diluted to contain approximately 1000 hemolytic units and 10,000 counts per ml. To 1 ml aliquot of the diluted eluates were added varying amounts of red cells.

† In more recent experiments, the globulin fraction used has been that precipitating between 33% and 40% saturation with $(NH_4)_2SO_4$. This fraction was found to be richest in hemolytic activity.

The mixtures were left at room temperature for 30 minutes and the cells separated by centrifugation. The absorption of hemolytic activity was determined by comparing the supernatants with the original eluate added to the cells. The absorption of radioactivity was determined by counting both the sediment and supernatant tubes. Two corrections were made to the observed counts in the sediment and in the supernatant tubes in calculating the percent labeled antibody absorbed.

The first correction was for the counts sedimented nonspecifically and for the supernatant counts left undecanted in the sediment tubes. The percentage of such counts was determined from a control tube which contained no red cells and usually approximated 5% of the counts in the supernatant. This percentage of the counts in each supernatant was then subtracted from the corresponding sediment count.

The second correction was for the fraction of radioactivity in the eluate not absorbable on excess red cells and assumed to be bound to nonantibody globulin. The reciprocal of this fraction served as a correction factor to convert the percent of total radioactivity absorbed on different amounts of red blood cells to per cent of total antibody absorbed. In summary, then, the percentage of total antibody absorbed from anti-Forssman eluates on different amounts of red blood cells or heated stromata was calculated as:

$$\frac{P-NS}{(P+S)F}\times 100,$$ the symbols standing for:

P = counts observed in sediment tube

S = counts observed in supernatant tube

P_e and S_e are counts observed in control sediment and supernatant tubes

$$N=\frac{P_e}{S_e}$$

F = fraction of radioactivity absorbable on excess red blood cells

Results are given in figures 1 and 2. The absorption of radioactivity from the first eluate (fig. 1) followed exactly the absorption of hemolytic activity. The second eluate, however, required more than 100 times the number of cells to absorb the radioactivity than was required to absorb the hemolytic activity (fig. 2). These results illustrate the importance of using in the primary absorption that amount of cells which is just sufficient to absorb most of the hemolytic activity. If this is done, the labeled antibody eluted from these cells will have absorption characteristics closely comparable to that of the hemolytic activity. With serums taken from rabbits after 2 to 3 weeks of immunization 6 units of cells in μg RBC Hgb per hemolytic unit were found to absorb more than 95% of the hemolytic activity. Serums obtained later in the course of immunization required much greater numbers of red cells. For this and other reasons to be discussed later, they are less satisfactory for making labeled antibody preparations.

Anti-Forssman competitive absorption test

The competitive absorption test described here requires the prior preparation of a labeled antibody eluate. Once prepared, this eluate may be used for several weeks to test a large number of serums. The eluate was prepared as previously described, using for labeling and elution a rabbit serum taken after 2 to 3 weeks immunization with heated stromata and using 6 units of red cells in μg RBC Hgb per hemolytic unit for the absorption of labeled antibody. Im-

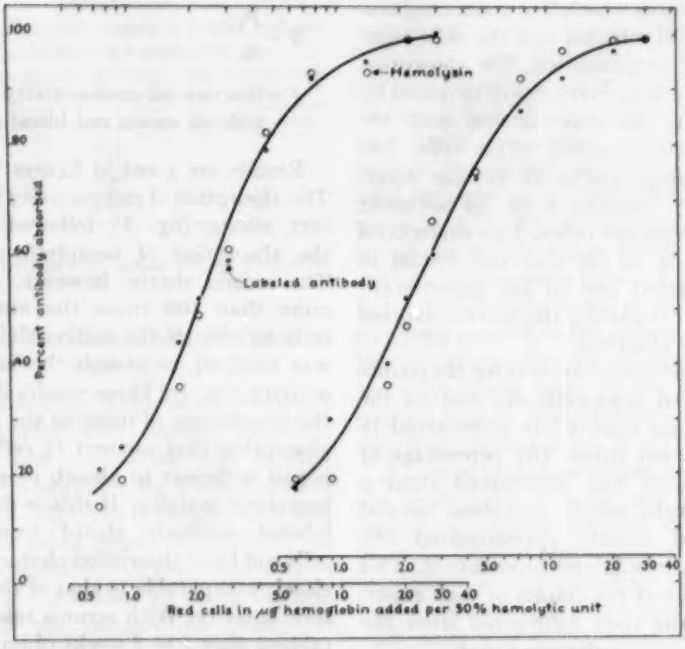

FIG. 1.—Absorption of hemolytic activity and radioactivity from first anti-Forssman eluate by varying numbers of sheep red cells. The results from two different eluates are shown.

mediately before use, the anti-Forssman eluate was clarified by centrifugation for 30 minutes in an average field of 600×*g* and was diluted with 0.6% BSA in 0.85% saline to contain 80 to 100 hemolysin units per ml. This is equivalent to approximately 0.2 combining units (see below) per ml, a factor which is important since a high combining activity in the eluate will decrease the sensitivity and precision of the test.

Preliminary and final determinations were usually made on each serum, the first with one or two tubes, the second with five. To each tube was added, in order, test serum* and saline to a combined volume of 0.6 ml, 0.5 ml diluted eluate and 0.5 ml of 0.4% sheep red cells (500 μg RBC Hgb). To each set was added a control tube which did not contain cells or test serum, but only eluate and saline diluent to a volume of 1.6 ml. The time and temperature were found not to be critical since the reaction approached equilibrium in a few minutes. However, the tubes were routinely agitated for 30 minutes at room temperature. They were then centrifuged for 10 minutes in an average field of 600×*g* and the supernatants decanted as completely as possible. Although no macroscopic agglutination occurred in the dilute red cell suspension, the cells packed firmly when centrifuged because of the definite antibody excess. After equalizing the volumes of the sediment and supernatant with water, these two tubes were counted in a well-type scintillation counter and the percent radioactivity

* Complement was inactivated by heating to 56 C for 45 minutes.

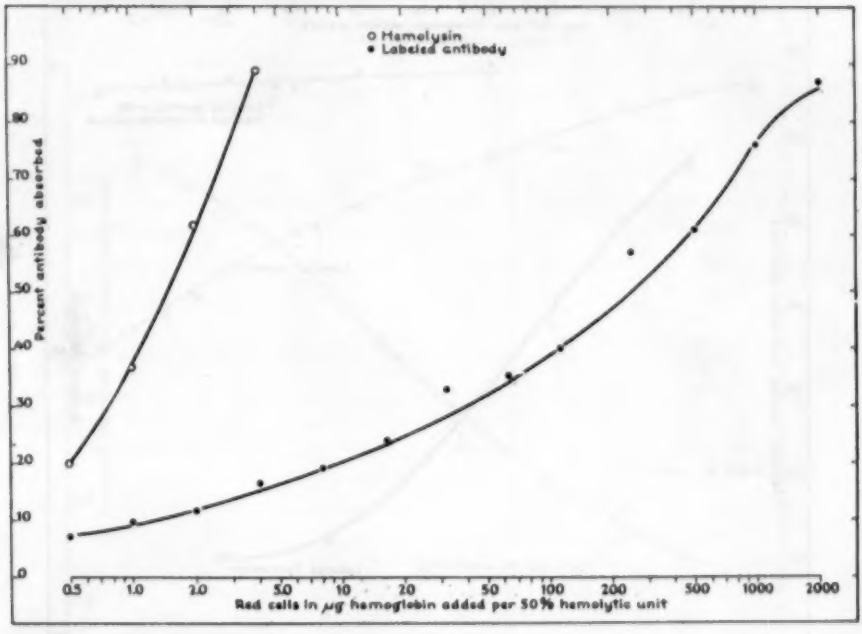

FIG. 2.—Absorption of hemolytic activity and radioactivity from second anti-Forssman eluate by varying numbers of sheep red cells.

absorbed was calculated. The control tube served as a test of the stability of the labeled antibody and a measure of the percentage of undecanted supernatant. This percentage usually approximated 5%.

Results with standard Forssman antiserum

Results obtained with an anti-Forssman serum are shown in the lower curve of fig. 3, in which the volume of serum is given on a log plot and the percent absorption on an arithmetic plot. When no serum was added, the uptake of radioactivity was 68%. When $10^{-2.1}$ (1/125) ml of the serum was added, the uptake of radioactivity was 34%, or exactly one-half of the uptake of eluate alone. This amount of serum was arbitrarily designated as containing one combining unit. This is the same serum used in the experiment described above to determine hemolytic activity per unit weight of antibody. The serum contained 38,000 hemolytic units per ml or 300 hemolytic units per combining unit. Since it has already been determined that with this serum 265 hemolytic units were equivalent to 1 μg antibody nitrogen, it follows that 1 combining unit is approximately 1 μg antibody nitrogen.

Table 4 shows the relationship between the number of cells used, the per-

TABLE 4.—*The effect of cell concentration on the sensitivity and precision of the competitive absorption test.*

Red cell concentration	Percent uptake of eluate alone	Dilution of serum producing 50% inhibition	Effective titer of Forssman antiserum
0.2%	53	$10^{-2.4}$	200
0.4%	68	$10^{-2.1}$	126
0.8%	74	$10^{-1.9}$	79

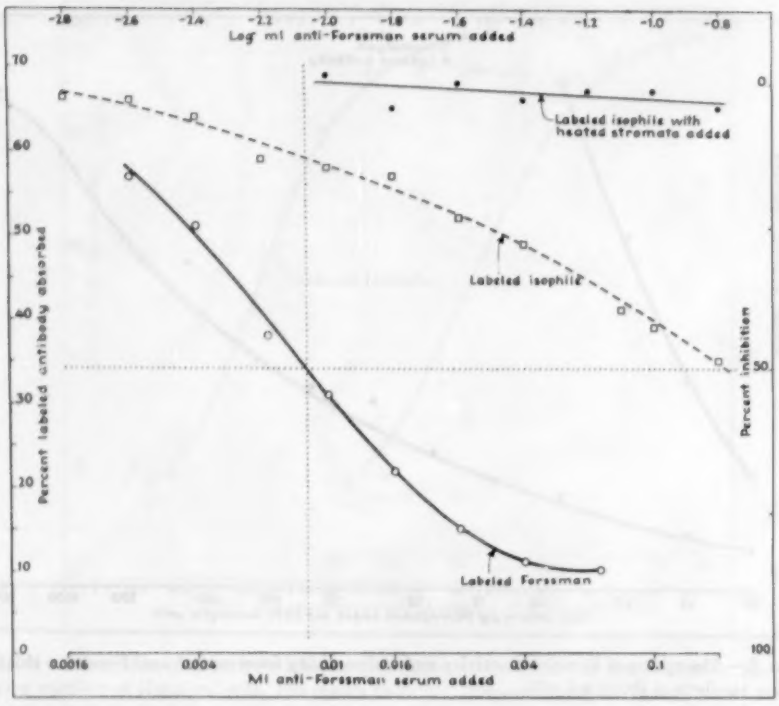

FIG. 3.—The effect of the standard anti-Forssman serum on the absorption by red cells of labeled Forssman antibody and labeled isophile antibody. For this experiment, the antiserum was diluted in saline.

cent uptake with eluate alone and the volume of Forssman antiserum required to produce 50% inhibition of uptake. While a decrease in the number of red cells used resulted in an increase in sensitivity of the test, the accompanying decrease in the labeled antibody taken up by the cells carried with it a loss of precision. The actual number of cells selected for the test (0.5 ml of 0.4% cells) is exactly one-tenth the number used in the standard hemolysin determination, a fact which facilitated comparison of the hemolytic and combining units. The cell suspension was sufficiently dilute to prevent a prompt agglutination of cells that might interfere with absorption of antibody.

Because of possible variation in antigen content of different batches of cells and in the avidity of antibody in different eluates, all subsequent determinations of combining units were made by comparison with the effect of one competitive unit (1/125 ml) of the anti-Forssman serum (38,000 hemolytic units per ml), which is hereafter referred to as the standard anti-Forssman serum. In actual practice, the effect of one combining unit of the standard serum rarely differed from 50% inhibition by more than 5%. The use of the standard comparison, however, obviated the necessity of making corrections for minor variations in experimental conditions and simplified the mathematical treatment of results since it also made unnecessary a correction for undecanted supernatant.

In preliminary experiments, the ef-

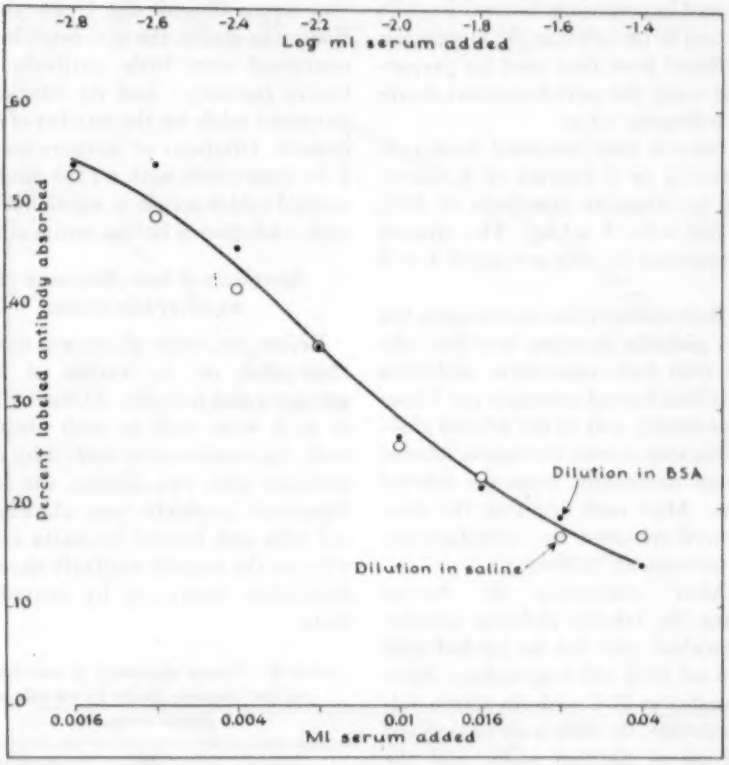

FIG. 4.—A comparison of the dilution of the standard anti-Forssman serum in saline and in bovine serum albumin on the competitive absorption curves. The eluate was diluted in saline.

fect of dilution of the standard anti-Forssman serum in saline and in normal rabbit serum was compared. These tests indicated that normal rabbit serum possessed a slight but definite blocking action on the uptake of labeled Forssman antibody. This was thought to be caused by the "natural" Forssman antibody in normal rabbit serum and attempts were made to absorb this antibody with sheep red cells. The use of such absorbed serums as diluents yielded erratic results, possibly due to the leaching of Forssman antigen from the red cells in the absorption process. Experiments were then performed using as a diluent for the standard anti-Forssman serum a 0.6% solution of bovine serum albumin in 0.85% saline. Figure 4 gives the results of a comparison of the effect of dilution in 0.85% saline and in bovine serum albumin. Since no significant difference could be detected, subsequent tests with anti-Forssman serums were made using 0.85% saline for the initial 1:10 dilution, and then a 0.6% solution of bovine serum albumin in saline for higher dilutions.

Anti-isophile eluate and competitive absorption test

The term isophile is used here to describe the heat labile antigens of the sheep red cell as opposed to the heat stable or Forssman antigens. The tech-

nique used in preparing the anti-isophile eluate and in performing the absorption test differed from that used for preparing and using the anti-Forssman eluate in the following ways:

(1) Serums were obtained from rabbits after 2 or 3 courses of 6 thrice-weekly intravenous injections of 10% sheep red cells, 1 ml/kg. The courses were separated by rest periods of 4 to 6 weeks.

(2) Before absorption on red cells, the labeled globulin fraction was first absorbed with two consecutive additions of one billion heated stromata per Forssman combining unit in the labeled globulin. This step served to remove labeled Forssman antibodies from the labeled globulin. After each addition the stromata were removed by centrifugation for 30 minutes at 20,000×*g*.

(3) After absorption by heated stromata the labeled globulin fraction was absorbed with 0.4 ml packed cells (from 4 ml 10% cell suspension). After three washes at 37 C with the serum-KI-saline mixture, the cells were hemolyzed with 5 ml of distilled water and the agglutinated stromata centrifuged at 20,000×*g*. The hemoglobin solution was decanted and 5 ml of normal rabbit serum added. The pH was brought to 3.2 by the careful addition of 0.2 *M* citric acid. The stromata were kept suspended at this pH at room temperature for 15 minutes and then removed by centrifuging for 30 minutes at 20,000×*g*. The supernatant containing the acid anti-isophile eluate was brought to a pH of 7.2 with 0.1 *N* NaOH.

(4) Before use, the acid isophile eluate was diluted in 1:5 normal rabbit serum* instead of 0.6% BSA in 0.85% saline. Normal rabbit serum had no detectable blocking effect on the labeled isophile antibody whereas bovine serum albumin did. Unlike the anti-Forssman eluate, the anti-isophile eluate contained very little antibody (combining capacity), and its dilution was governed solely by the number of counts desired. Dilutions of antiserums above 1:10 were made with a 1:10 dilution of normal rabbit serum in saline instead of with a solution of bovine serum albumin.

* Complement was inactivated by heating at 56 C for 45 minutes.

Specificity of anti-Forssman and anti-isophile eluates

Before use, every eluate was tested by absorption on an excess of heated stromata and red cells. At least 1 billion of each were used in such absorption tests. As illustrated in table 5 by results obtained with two eluates, the labeled Forssman antibody was absorbed by red cells and heated stromata equally, whereas the isophile antibody showed no detectable absorption by heated stromata.

TABLE 5.—*Percent absorption of anti-Forssman and anti-isophile eluates by red cells and heated stromata.*

Eluate	RBC	Heated stromata
Anti-Forssman	90	88
Anti-isophile	86	0

Cross-blocking action of Forssman and isophile antibodies

Despite distinct differences in specificity as indicated in table 5, the Forssman and isophile antibodies have been shown to have a cross-blocking effect, i.e., they are able to block the uptake of the labeled antibody of different specificity (cross-blocking) as well as that of the same specificity (specific blocking).

The effect of the standard anti-Forssman serum in blocking the uptake of the labeled isophile antibody is compared in figure 3 with the ability of the same serum to block the uptake of labeled Forssman antibody. Figure 5 gives the results obtained with a serum

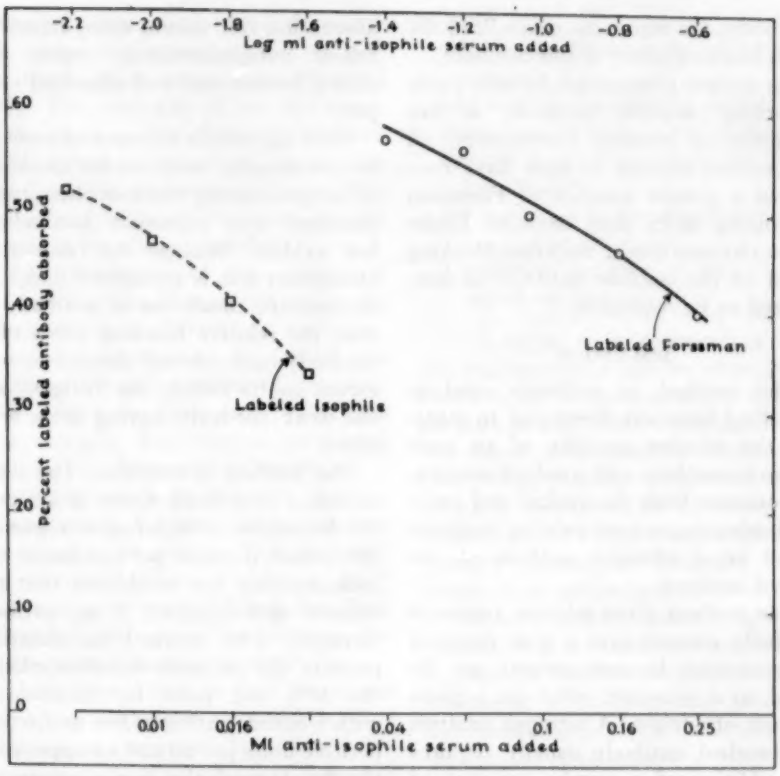

FIG. 5.—The effect of an anti-isophile serum on the absorption by red cells of labeled isophile antibody and labeled Forssman antibody. The antiserum and Forssman eluate were diluted in saline.

containing isophile antibody, from which Forssman antibody had been absorbed by heated stromata and the stromata removed by centrifugation for 200 minutes at 105,000×*g*. Two differences between the specific and cross-blocking curves are noted. As compared to the cross-blocking curve, the specific blocking curve had a steeper slope and requires less than one-tenth the amount of serum for 50% inhibition of uptake.

When the anti-Forssman serum was absorbed with heated stromata, the cross-blocking of the uptake of labeled isophile antibody disappeared. This indicates that the cross-blocking effect was not produced by the presence in the anti-Forssman serum of antibody of isophile specificity.

From the cross-blocking effects demonstrated above, it is apparent that when both Forssman and isophile antibodies are present, the one having the greater number of combining units will influence the results obtained with the other labeled antibody. To measure the isophile combining activity in anti-red cell serums, a concentrated heated stromata suspension containing 16×10^9 cells/ml was added to the serum dilutions before the addition of red cells. For each Forssman combining unit present, 0.02 ml of the stromata suspension was added. By absorbing the Forssman

antibody, the stromata neutralized the cross-blocking effect of this antibody.

An antigen preparation for selectively absorbing isophile antibody is not available at present. Fortunately, all the serums studied to date have contained a greater number of Forssman combining units than isophile. Under these circumstances, the cross-blocking effect of the isophile antibody is considered to be negligible.

DISCUSSION

The method of antibody analysis described here was developed to measure the relative capacity of an antiserum to combine with a red cell antigen. It possesses both theoretical and practical advantages over existing methods based on a nitrogen analysis of absorbed antibody.

The method gives relative values of antibody content over a wide range of concentration because serums are diluted to a constant effect on a given amount of antigen. A nitrogen analysis of absorbed antibody usually requires the addition of a varying amount of antigen to a constant volume of serum. With an equilibrium reaction such as that between antigen and antibody, a change in the concentration of the reactants will effect the state of equilibrium. At high concentrations of antibody the error produced by this change is small, but at low concentrations of antibody the error may be considerable.[17]

Instead of measuring the total weight of antibody bound to antigen, the competititve absorption test measures the relative ability of antibody to block antigen sites. Since antibodies are known to vary in molecular size, and may vary in valence, the competitive absorption test should more accurately reflect antigen-antibody union than does a measurement of absorbed nitrogen.

Since agglutinin nitrogen absorptions are necessarily made under conditions of antigen excess, much of the nitrogen absorbed may represent antibody of low avidity. Because the competitive absorption test is performed under the competitive conditions of antibody excess, the relative blocking effect of an antibody molecule will depend to some extent on its avidity for antigen, with the weak antibody having little or no effect.

The method is sensitive. The determination on a single serum of a ratio of 265 hemolytic units per μg nitrogen and 300 hemolytic units per combining unit indicates that one combining unit represents approximately 1 μg antibody nitrogen. The method as described permits the accurate determination of the 50% end point by interpolation with a concentration as low as 1.6 competitive units per ml and an approximation by extrapolation to a concentration as low as 1 unit per ml. The ratio of 1 combining unit per μg N can be taken only as an order of magnitude, since it is unlikely that there is a completely constant ratio between these two units.

The competitive absorption test can be applied to antibodies of a single specificity even though the insoluble particle is like the red cell, a mixture of antigens. This is because the labeled antibody is separated under conditions of considerable antibody excess, and as a consequence antibody specific for minor antigenic groupings of the red cell will have relatively little representation in the eluate. Since unlabeled antibody specific for another major antigenic group has a blocking effect less than 10% as great as that with the same specificity as the labeled antibody, it does not seem likely that antibody

17. Talmage, D. W. and Maurer, P. H. 1953, I^{131} labeled antigen precipitation as a measure of quantity and quality of antibody. J. Infect. Dis. 92: 288–300.

directed against a less frequently occurring antigenic grouping of the cell surface would have a significant blocking effect. The similarity of the two cross-blocking curves (fig. 3 and 4) suggests that the two major antigenic groupings (the Forssman and isophile) occur with approximately equal frequency on the surface of the sheep red cell.

The "blocking" of the uptake of of labeled antibody does not imply the presence of a "blocking antibody" such as that proposed by Cooke and Loveless.[18,19] All antibodies are considered to have a capacity to combine with antigen. The relative combining capacity of the serum is expressed in combining units to indicate that its measurement is based on the physical combination of antigen and antibody. An alternative term "blocking unit" was originally used,[12,20] but has been discarded because of its obvious confusion with "blocking antibody."

Using the ratio of 265 Forssman hemolytic units per μg nitrogen, it is possible to estimate that there are approximately 49 molecules of Forssman antibody per red cell at the end point of the hemolytic titrations (50% hemolysis in 30 minutes).

Molecules of antibody

$$= \frac{(6.25)(6.06\times10^{23})}{(265)(900{,}000)(\times10^{6})(3.2\times10^{8})} = 49$$

Where

6.25 is μg protein per μg protein nitrogen,

6.06×10^{23} is Avogadro's number

265 is hemolytic units per μg nitrogen

900,000 is gram molecular weight of hemolysin[21]

10^{6} is placed in the denominator to convert grams to μg

3.2×10^{8} is number of red cells in hemolytic titration

This figure may represent a more efficient hemolytic activity than Mayer et al's figure of 48 molecules of hemolysin per red cell,[21] since their calculation was based on the number of molecules of antibody required to produce nearly complete hemolysis in 7½ hours. The actual number of molecules required to produce hemolysis probably varies widely from serum to serum.[5]

The serum with which the above calculation was made was found to have a ratio of 300 hemolytic units per combining unit. Since the hemolysin determination is made with ten times the number of cells used in the determination of combining units, the ratio of antibody molecules to red cell at the end point of the absorption test is $300\times10\times49 = 147{,}000$. At this point most of the Forssman antigen sites on the red cell are occupied, while only half the antibody molecules are absorbed on the red cell. Thus, the number of Forssman antigen sites per red cell is approximately one-half of 147,000 or 73,500.

The relationship between the number of antibody molecules and the number of antigen sites is important in any study of the mechanism of hemolysis. The values of 49 molecules and 73,500 antigen sites per cell should be considered limiting values since they are based on the assumption that the molecular weight of

18. Cooke, R. A., Barnard, J. H., Hebald, S. and Stull, A. 1935, Serological evidence of immunity with coexisting sensitization in a type of human allergy (hay fever). J. Exper. Med. **62**: 733–750.

19. Loveless, M. H. 1940, Immunological studies of pollinosis. I. The presence of two antibodies related to the same pollen-antigen in the serum of treated hay fever patients. J. Immunol. **38**: 25–50.

20. Talmage, D. W., Freter, G. G. and Taliaferro, W. H. 1955, A comparison of the hemolytic and blocking activities of antisheep red cell serums. J. Lab. & Clin. Med. **46**: 959.

21. Mayer, M. M., Croft, C. C. and Gray, M. M. 1948, Kinetic studies on immune hemolysis. I. A method. J. Exper. Med. **88**: 427–444.

the antibody is 900,000. The results presented in the next two papers indicate that anti-Forssman serums contain two antibodies: a heavier hemolytic molecule and a lighter relatively nonhemolytic molecule. The effect of the second molecule will be to lower the average molecular weight and increase the calculated number of antigen sites per red cell. The total number of both types of antibody molecules present when 50% hemolysis occurs in 30 minutes is probably greater than 49 per cell, and the number of the hemolytic molecules is probably less than 49 per cell.

SUMMARY

1. A method developed for measuring the relative combining capacities of antisheep red cell serums is based on the blocking of the uptake by the cells of an I^{131} trace-labeled antibody. The serums tested are not labeled with I^{131}, but compete with or block a labeled antibody which has been prepared and standardized beforehand. This manipulation has two advantages: first, one labeled antibody preparation may be used to test a large number of serums, and second, antibody to be titrated is not labeled.

2. The end point chosen for measuring combining capacity is that amount of serum producing 50% inhibition of uptake of labeled antibody. Since this point occurs under the competitive conditions of antibody excess, the relative blocking effect of an antibody molecule will depend to some extent on its avidity for antigen. As compared to the measurement of absorbed nitrogen, the competitive absorption test is more sensitive and simpler to perform. Relative values are more accurate since serums are diluted to a constant antibody concentration.

3. Methods have been described for separating labeled antibodies which are specific for each of the two major antigenic groups of the red cell, the heat stable or Forssman antigen and the heat labile or isophile. Antiserums reactive with only one of the antigens were shown to block the uptake of labeled antibody specific for the other, but this cross-blocking reaction was less than 10% as efficient as the specific blocking reaction.

THE EFFECT OF REPEATED INJECTIONS OF SHEEP RED CELLS ON THE HEMOLYTIC AND COMBINING CAPACITIES OF RABBIT ANTISERUMS

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The usual response of an animal to a second injection of an antigen is a more rapid and sometimes greater production of antibody than that occurring following the first injection. However, under certain circumstances large or repeated injections of an antigen into an animal reduce the ability of that animal to produce antibodies to the same antigen. Felton[1] used the term "immunologic paralysis" to describe the lasting inability of mice to respond to pneumococcal polysaccharides after they were given a large injection of the same antigen. Dixon, Maurer and Weigle[2] have demonstrated that mice so treated with pneumococcal polysaccharides metabolize passively transferred antibody faster than do controls. This gave support to a previous suggestion that persisting antigen might be neutralizing antibody as it was formed.[3] W. H. and L. G. Taliaferro[4] found a temporary "refractory phase" in the hemolysin production of rabbits given repeated injections of sheep red cells. After reaching peak levels, hemolytic titers followed a logarithmic decline which generally continued unchanged for a month or more following the cessation of the repeated injections of red cells. This refractoriness could not be explained by the neutralization of antibody by antigen, since the animals possessed a large excess of circulating antibody on which the injection of red cells produced only a negligible decrease. In addition, they found by a technique of splenic transfer[5] that the spleen lost its antibody-forming capacity during the period of serum antibody decline that occurs following the repeated injection of antigen.

Dixon and Maurer[6] studied the immune response of rabbits to large and repeated injections of heterologous serum proteins and described two types of "immunologic unresponsiveness." In adult rabbits, they found a temporary unresponsiveness which resembled the refractory phase of W. H. and L. G. Taliaferro in that (1) recovery occurred in a period of months, and (2) neutralization by residual antigen could not

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1. Felton, D. D. 1941, The significance of antigen in animal tissues. J. Immunol. **61**: 107–117.
2. Dixon, F. J., Maurer, P. H. and Weigle, W. O. 1955, Immunologic activity of pneumococcal polysaccharide fixed in the tissues of the mouse. J. Immunol. **74**: 188–191.
3. Kaplan, M. H., Coons, A. H. and Deane, H. W. 1949, Localization of antigen in tissue cells. III. Cellular distribution of pneumococcal polysaccharides types II and III in the mouse. J. Exper. Med. **91**: 15–29.
4. Taliaferro, W. H. and Taliaferro, L. G. 1951, The role of the spleen in hemolysin production in rabbits receiving multiple antigen injections. J. Infect. Dis. **89**: 143–168.
5. Taliaferro, W. H. 1955, Studies in antibody formation. Some Physiological Aspects and Consequences of Parasitism, Rutgers University Press, W. H. Cole, ed. pp. 50–75.
6. Dixon, F. J. and Maurer, P. H. 1955, Immunologic unresponsiveness induced by protein antigens. J. Immunol. **101**: 245–257.

explain the failure to develop circulating antibody. Dixon and Maurer also described a permanent unresponsiveness which occurred when the antigen was given to newborn rabbits or to rabbits after whole body X radiation. This type of unresponsiveness was thought due to an "acceptance of the foreign protein as non-antigenic by the host." It is probably similar to other instances of lasting unresponsiveness which have been reported to follow exposure of embryos or new born animals to antigen.[7,8]

Antigen-induced immunologic refractoriness is specific for the antigen injected. During both the temporary and permanent unresponsiveness of Dixon and Maurer's experiment, the animals were able to respond normally to other antigens. In experiments reported in the present paper using the isophile and Forssman antigens of the sheep red cell, the specificity of immunologic refractoriness is confirmed. These experiments also suggest the production of two antibodies directed against the same antigen (Forssman). One of these antibodies reaches peak production during the refractory phase of the other.

EXPERIMENTAL PROCEDURE AND RESULTS

Thrice weekly for 6 weeks, 6 rabbits were given intravenous injections of 1 ml/kg of 10% sheep red cells. Each week, 3 days after the last injection, 10 ml were bled from the ear vein of each rabbit. Serums were separated and analyzed for hemolytic units,[9] Forssman combining units and isophile combining units. The last two determinations were based on the blocking of the uptake of labeled antibody as described in the preceding paper.[10] Isophile determinations were made both with and without the addition of heated stromata. Averaged results are given in table 1 and figure 1.

Peak hemolysin titer was reached in 5 rabbits by the end of the 2nd week and in 1 rabbit by the 3rd week. During the 4th week, the hemolysin titer fell sharply in every animal. The decrease in titer during this 7-day period averaged 38% of the 3rd week titer. By the 6th week, hemolytic activity had decreased to between one-third and one-fifth peak levels.

Forssman combining titers reached a peak level in 4 rabbits by the 4th week and then decreased slightly by the 6th week. In 2 rabbits Forssman combining titers rose each week for the 6 weeks of the experiment.

Isophile combining titers obtained by the addition of heated stromata remained below significant levels until the 3rd week and could not be measured in all animals until the 4th week. In 3 animals the isophile titers decreased between the 4th and 6th weeks while in 2 they increased.

The serums obtained at the 3rd, 4th and 6th weeks were absorbed twice with a heated stromata suspension, using for each Forssman combining unit 0.02 ml of a suspension containing 16.3×10^9 cells per ml. Since the second absorption produced relatively little change in hemolysin titer, the un-

7. Snell, G. D. 1952, The immunogenetics of tumor transplantation. Cancer Res. **12**: 543–546.

8. Billingham, R. E., Brent, L. and Medawar, P. B. 1953, Actively acquired tolerance of foreign cells. Nature **172**: 603–606.

9. Taliaferro, W. H. and Taliaferro, L. G. 1950, The dynamics of hemolysin formation in intact and splenectomized rabbits. J. Infect. Dis. **87**: 37–62.

10. Talmage, D. W. and Freter, G. G., 1956, The blocking of labeled antibody uptake as a measure of the combining capacity of anti-red cell serums. J. Infect. Dis. **98**: 277–292.

TABLE 1.—*Relationship of hemolytic, Forssman combining and isophile combining titers in serums of 6 rabbits injected thrice weekly with 10% sheep red cells.*

Weeks of immunization		Total hemolytic units	Percent absorbed on heated stromata	Forssman combining units	Isophile combining units		Hemolytic efficiency	
					Without stromata	With stromata	Forssman	Isophile
1	Mean	12,260		19.6	0.8		611	
	S.E.	2,400		3.5	0.4		75	
2	Mean	26,000		64.8	3.8		411	
	S.E.	4,100		7.8	0.9		40	
3	Mean	29,000	96.3	109.5	23.5		245	
	S.E.	7,400	0.8	17.6	6.5		53	
4	Mean	14,480	93.4	193.6	77.2	38.4	98	35
	S.E.	3,900	1.9	55.1	30.8	15.1	24	11
6	Mean	7,420	88.4	148.0	39.0	24.0	64	27
	S.E.	1,100	6.7	42.3	4.8	3.5	42	9

S.E., standard error.
Hemolytic efficiency is the ratio of hemolytic to combining units.

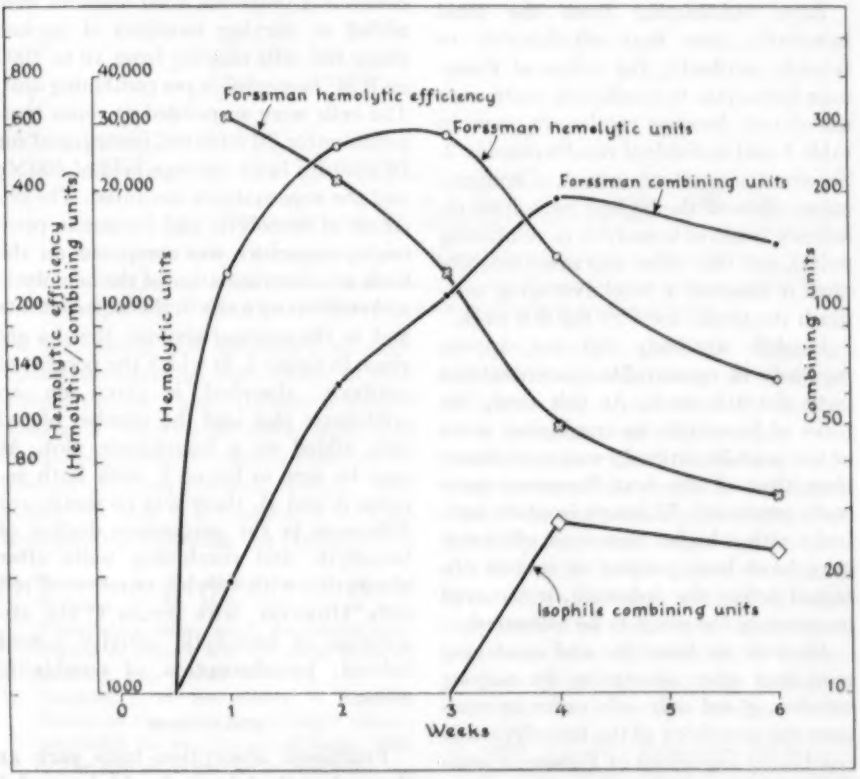

FIG. 1.—Relationship of hemolytic, Forssman combining and isophile combining titers in serums of 6 rabbits injected thrice weekly with 10% sheep red cells.

TABLE 2.—*Hemolytic efficiency of serums obtained after thrice weekly injections of 10% sheep red cells into rabbits.*

Weeks of immunisation	Animal no.						Mean and S.E.
	1	2	3	4	5	6	
1	941	440	679	470	619	519	611 ± 75
2	472	400	396	536	381	284	411 ± 40
3	271	456	184	216	169	201	245 ± 53
4	71	185	33	155	57	87	98 ± 24
6	77		26	129	27	59	64 ± 42

S.E., standard error.
Hemolytic efficiency is the ratio of hemolytic to combining units.

absorbed hemolytic activity was attributed to isophile antibody. The fraction of hemolytic activity attributed to isophile antibody as a result of these absorptions increased between the 3rd and 6th weeks.

After subtracting from the total hemolytic titer that attributable to isophile antibody, the ratios of Forssman hemolytic to combining units were calculated. Average results are given in table 1 and individual results in table 2. Serums drawn after one week of immunization showed the highest hemolytic efficiency (ratio of hemolytic to combining units), and this value decreased steadily until it reached a level averaging one-ninth the initial level by the 6th week.

Isophile antibody did not appear regularly in measurable concentrations until the 4th week. At this time, the ratio of hemolytic to combining units of the isophile antibody was much lower than that of the first Forssman antibody produced. Although isophile antibody with a higher hemolytic efficiency may have been present in serums obtained before the 3rd week, it occurred in amounts too small to be measured.

Decrease in hemolytic and combining capacities after absorption by varying numbers of red cells.—In order to compare the specificity of the hemolytic and combining capacities of Forssman antiserums, serums with high and low ratios of these two activities were absorbed with varying numbers of red cells. For this purpose, 3 serums were used. The first (serum A) was the anti-Forssman serum used as a standard for all competitive absorption tests. This serum contained a relatively high ratio of 300 hemolytic units per combining unit.

The other 2 serums (B and C) were obtained after 6 weeks of thrice weekly injections of 10% sheep red cells. Although some isophile antibody was present in these 2 serums, it amounted to less than 10% of the hemolytic activity and less than 12% of the combining capacity. The 2 serums contained ratios of 26 and 27 hemolytic units per Forssman combining unit. After the serums* were diluted to contain 50 combining units/ml, 1 ml aliquots were added to varying numbers of packed sheep red cells ranging from 10 to 1000 μg RBC hemoglobin per combining unit. The cells were suspended at room temperature for 20 minutes, centrifuged for 10 minutes in an average field of $600 \times g$ and the supernatants decanted. The decrease of hemolytic and Forssman combining capacities was compared on the basis of a determination of the hemolytic and combining units in the supernatants and in the original serums. Results are given in figure 2, in which the percent of antibody absorbed is given on an arithmetic plot and the number of red cells added on a logarithmic plot. As may be seen in figure 2, with both serums A and B, there was no significant difference in the percentage decline of hemolytic and combining units after absorption with varying numbers of red cells. However, with serum C the absorption of hemolytic activity lagged behind the absorption of combining units.

DISCUSSION

Fractional absorption tests such as those described here should be useful

* Complement was inactivated by heating to 56 C for 45 minutes.

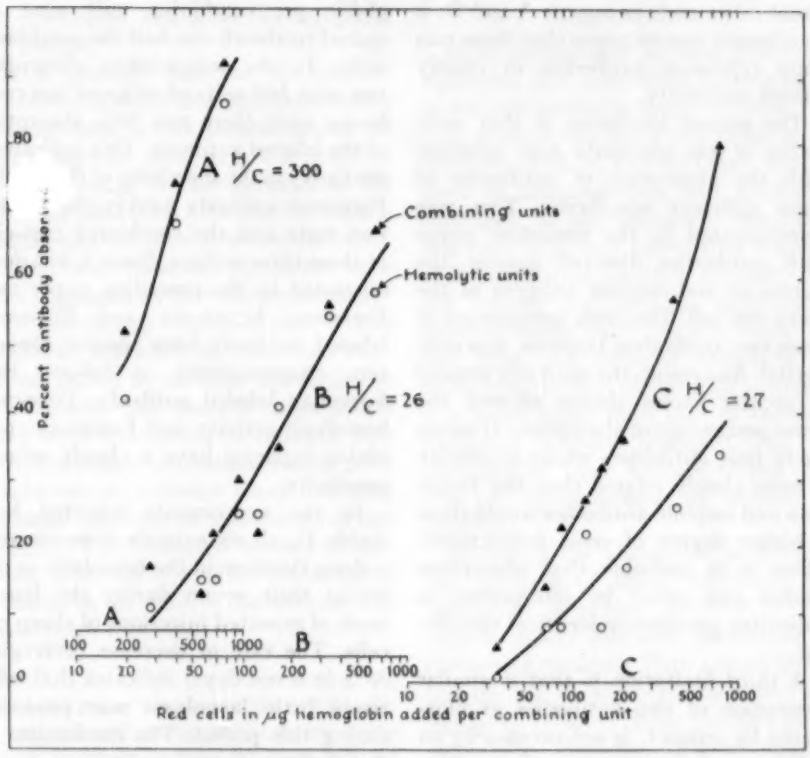

FIG. 2.—Comparison of the absorption of hemolytic and Forssman combining capacities by red cells from serums with (A) a high and (B and C) a low hemolytic to combining ratio.

for comparing the specificity of antibodies manifesting themselves in two different ways. These absorption studies possess a theoretical advantage over similar precipitation studies reported by Sherman,[11] Vaughan and Kabat,[12] and others[13] in that it is frequently difficult to determine following the precipitation of the antigen-antibody aggregate whether nonprecipitating antibody remains in the supernatant. Absorption tests using an insoluble antigen avoid this difficulty entirely. However, the interpretation of the results of the absorption (or precipitation) of a single serum is subject to several other limitations.

The first limitation is that the absorption of two different antibodies might conceivably be equal in a single serum as a fortuitous circumstance. The similar absorption of hemolytic and combining units from two serums of dif-

11. Sherman, W. B., Menzel, A. E. O. and Seebohm, P. M. 1950, The role of nonprecipitating antibodies in the passive sensitization of human skin by rabbit antiovalbumin. J. Exper. Med. 92: 191-200.

12. Vaughan, J. H. and Kabat, E. A. 1953, Studies on the antibodies in rabbit antisera responsible for sensitization of human skin. I. The role of impurities in crystalline egg albumin in stimulating the production of skin sensitizing antibody. J. Exper. Med. 97: 821-844.

13. Rapp, H. J. 1953, Purification and immunochemical characterization of the heat stable, alcohol-soluble hemolytic antibody inhibitor of the sheep erythrocyte. Thesis, Johns Hopkins University.

ferent ratio, such as serums A and B, is a necessary step to prove that these two units represent antibodies of closely related specificity.

The second limitation is that antibodies of one specificity may interfere with the absorption of antibodies of quite different specificity. This was demonstrated in the preceding paper with antibodies directed against the Forssman and isophile antigens of the sheep red cell. The cross interference of these two antibodies, however, was only partial. As a result, the antibody present in greater excess always showed the lower percentage of absorption. It seems likely that antibodies whose specificity is more closely related than the Forssman and isophile antibodies would show a higher degree of cross interference. Thus, it is probable that absorption studies can never be interpreted as indicating completely identical specificity.

A third limitation is that dissimilar absorption of two antibodies as illustrated by serum C is not necessarily an indication of dissimilar specificity. This is because other factors such as size or avidity might conceivably affect the degree of absorption of antibody. If the hemolytic and combining units represented two antibodies of different specificity, then in serums B and C, with a relatively greater number of combining units than serum A, the absorption of combining units should have lagged behind the absorption of hemolytic activity. Actually, the reverse was true in serum C. Thus, the disparity in the absorption occurring in serum C cannot be explained on the basis of two antibodies of dissimilar specificity, but is more probably due to the selective absorption of avid antibody of low hemolytic efficiency.

In all three serums, approximately 500 units of red cells in μg RBC hemoglobin per combining unit were required to absorb one-half the combining units. In the competititve absorption test with 500 units of cells and one combining unit, there was 50% absorption of the labeled antibody. This indicates a similarity in the specificity of the labeled Forssman antibody used in the absorption tests and the combining capacity of these three serums. Since it was demonstrated in the preceding paper that Forssman hemolysin and Forssman labeled antibody have similar absorption characteristics, it follows that Forssman labeled antibody, Forssman hemolytic activity and Forssman combining capacity have a closely related specificity.

In the experiments reported here (table 1), all six animals demonstrated a sharp decrease in the hemolytic activity of their serum during the fourth week of repeated injections of sheep red cells. The rate of decrease (averaging 38% in seven days) indicates that relatively little hemolysin was produced during this period. The mechanism of this "refractoriness" is not clear.

During the fourth week when the production of hemolytic units was declining, isophile combining capacity as measured by the competitive absorption test became measurable in all rabbits for the first time. In three animals it reached a peak level at this time. This indicates that the refractoriness observed in hemolytic titers did not represent a general decrease in all antibody production.

More difficult to understand is the rise in Forssman-combining capacity during the period of sharpest drop of Forssman hemolytic activity. Since these two activities are absorbed nearly equally by red cells, the drop in hemolytic activity cannot be simply an absorption by the injected red cells. Nor can it be an inability to recognize

the Forssman substance as antigenic or "foreign."

It is possible that the decrease in hemolytic activity does not represent a real decrease in the production of an anti-Forssman antibody but only a gradual change in the character of the antibody produced. However, in experiments reported elsewhere two distinctly different anti-Forssman antibodies have been separated by both electrophoretic[14] and centrifugal fractionation.[15] The hemolytic efficiency of these two antibodies differs by at least 100-fold. For this reason, the present experiments have been interpreted to indicate that two Forssman antibodies reach peak production at different times following repeated injection of the sheep red cell antigen. The changing hemolytic efficiency of the serums appears to be due to a change in the relative concentration of these two antibodies.

14. Stelos, P. and Talmage, D. W. 1956, Electrophoretic and ultracentrifugal studies of rabbit antiserum to sheep red blood cells and to boiled sheep RBC stromata. Fed. Proc. **15**: 615.

15. Talmage, D. W., Freter, G. G. and Taliaferro, W. H. 1956, Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation. J. Infect. Dis. **98**: 300–305.

SUMMARY

In a comparison of the Forssman hemolytic and combining capacities of rabbit antiserums to sheep red cells, the ratio of these two titers was found to be relatively high in serums taken after 1 week of immunization and gradually decreased to approximately one-ninth this ratio in serums taken after 6 weeks of immunization. Isophile antibody did not appear regularly in measurable concentrations until the 4th week. At this time, the ratio of hemolytic to combining units of the isophile antibody was much lower than that of the first Forssman antibody produced. The implications of these findings to the concept of refractoriness to antigen is discussed.

Following the absorption with varying numbers of sheep red cells, the decline in hemolytic activity in two serums closely followed the decline in combining capacity. With a third serum the decline in hemolytic activity was significantly lower than the decline in combining capacity. These findings taken together with those of the following paper[15] have been interpreted as indicating the presence in these serums of two Forssman antibodies which have closely related specificities, but differ in hemolytic efficiency.

TWO ANTIBODIES OF RELATED SPECIFICITY BUT DIFFERENT HEMOLYTIC EFFICIENCY SEPARATED BY CENTRIFUGATION

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In a comparison of the Forssman hemolytic and the combining capacities of rabbit antisheep red cell serums,[1] the ratio of these two titers was found to be relatively high in serums taken after 1 week of immunization, and gradually decreased to approximately one-ninth this ratio in serums taken after 6 weeks of immunization. This change in hemolytic efficiency of the antiserums suggested the possibility that two different antibodies might be present and that the changes noted were due to a change in the relative amounts of the two antibodies produced as immunization progressed.

Zinsser[2] and others[3] have demonstrated that the same antibody can manifest itself in a number of different ways, e.g., by precipitation, agglutination and complement fixation. These experiments effectively disproved previously held concepts that every manifestation of antibody activity was a function of a different type of antibody molecule, but did not answer the question of why some antibodies perform a given function more efficiently than do other antibodies. Opinion on this basic question is divided. One concept currently held is that all known differences in antibody activity can be explained on the basis of the varying specificities and reactivities of the antibodies involved. Opposing this concept is the view that some of the variations in antibody activity are due to differences in the physical properties of the antibody molecules. Two examples which illustrate this controversy are the Rh blocking antibodies and the skin-sensitizing antibodies (called "reagins" by some proponents of the second viewpoint).

Under certain circumstances human serum may contain antibodies which will block the agglutination of Rh positive human red cells by anti-Rh agglutinating serum. Although the agglutinating and blocking antibodies have different physical properties,[4] it does not necessarily follow that this physical difference is the cause of the difference in antibody manifestation. The agglutination of trypsinized red cells by blocking antibody[5] has been cited as indicating a difference in specificity between agglutinating and blocking

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1. Talmage, D. W., Freter, G. G. and Taliaferro, W. H. 1956, The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums. J. Infect. Dis. **98**: 293–299.
2. Zinsser, H. 1930, Note on the quantitative relations of antigen and antibody in agglutination and precipitation reaction. J. Immunol. **18**: 483–486.
3. Jones, F. S. 1927, Agglutination by precipitins. J. Exper. Med. **46**: 303–314.
4. Hill, J. M., Haberman, S. and Guy, R. 1949' Further evidence for antibodies of third order· Fractionation of agglutinins, blocking antibodies and cryptagglutinoids by physicochemical methods. Am. J. Clin. Path. **19**: 134–140.
5. Morton, J. A. and Pickles, M. M. 1947, Use of trypsin in the detection of incomplete Rh antibodies. Nature, **159**: 779.

antibodies.[6] The cross-blocking of Forssman and isophile antibodies illustrates the fact that the blocking of one antibody by another cannot be used as evidence of identity of specificity.[7] It is impossible to be certain at the present time that the variation in the physical properties of the Rh antibodies plays any role in their varied manifestation.

A somewhat similar situation exists with skin-sensitizing antibodies which, in general, have been found in the β or the γ_1 globulin fractions of human serum.[8] In an electrophoretic study of diphtheria antitoxins, Kuhns found precipitating antitoxin in the γ_2 fraction and nonprecipitating antitoxins in the γ_1 fraction.[9] The ability of both antibodies to neutralize toxin was considered as evidence of identity of specificity. Only the nonprecipitating antitoxin could sensitize human skin.[10] Both antitoxic and skin-sensitizing activities persisted for many days at the site of injection of nonprecipitating antitoxin, but precipitating antitoxin diffused away rapidly from the site of injection. These experiments have not been universally accepted as proving that the different manifestations of antibody activity are due to differences in the physical properties of the antibodies. It is remotely possible that the skin reactions were induced by a contaminant allergen in the toxin preparation.[11,12] It is also possible that the same toxin is neutralized in different ways by two antibodies of different specificity. This might occur if the combining site of one of the antibodies were directed toward the tissue-cell-toxin compound instead of the toxin molecule alone. Such a possibility is suggested by Ackroyd's demonstration of an antibody directed against the sedormid-platelet complex[13] and Eisen's finding of three specificities of antibodies in the serums of rabbits immunized with a hapten-protein compound.[14] An anti-

6. Kabat, E. A. 1951, The unity and diversity of antibodies, in The Nature and Significance of The Antibody Response, Edited by A. M. Pappenheimer, Jr. Symposium. N. Y. Acad. Med., Columbia University Press, New York.
7. Talmage, D. W. and Freter, G. G. 1956, The blocking of labeled antibody uptake as a measure of the combining capacity of antired cell serums. J. Infect. Dis. **98**: 277–292.
8. Sehon, A. H., Fyles, T. W. and Rose, B. 1955, Electrophoretic separation of skin-sensitizing antibody from the sera of ragweed-sensitive patients. J. Allergy, **26**: 329–339.
9. Kuhns, W. J. 1954, Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. IV. Differences between human precipitative and nonprecipitative skin-sensitizing diphtheria antitoxin as shown by electrophoresis. J. Exper. Med. **99**: 577–588.
10. Kuhns, W. J. and Pappenheimer, A. M., Jr. 1952, Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. I. Relationship of skin sensitivity to purified diphtheria toxoid to the presence of circulating, non-precipitating antitoxin. II. A comparison between the immunological properties of precipitating and non-precipitating (skin-sensitizing) antitoxins. J. Exper. Med. **95**: 363–392.
11. Kuhns, W. J. 1955, Immunochemical studies of antitoxin produced in normal and allergic individuals hyperimmunized with diphtheria toxoid. VI. Further investigations on the identity and specificity of non-precipitating skin-sensitizing antitoxin. J. Exper. Med. **101**: 109–117.
12. Vaughan, J. H. and Kabat, E. A. 1953, Studies on the antibodies in rabbit antisera responsible for sensitization of human skin. I. The role of impurities in crystalline egg albumen in stimulating the production of skin sensitizing antibody. J. Exper. Med. **97**: 821–844.
13. Ackroyd, J. F. 1949, The pathogenesis of thrombocytopenic purpura due to hypersensitivity to Sedormid (Allyl-isopropyl-acetyl-carbonide.) Clin. Sc. **7**: 249.
14. Eisen, H. N., Carsten, M. E. and Belman, S. 1954, Studies of hypersensitivity to low molecular weight substances. III. The 2,4-dinitrophenyl group as a determinant in the precipitin reaction. J. Immunol. **73**: 296–308.

body directed against both the toxin and the cellular components of the tissue-cell-toxin compound might be expected to have (like the skin-sensitizing antibody) more affinity for the cell and less affinity for the toxin alone than would an antibody with a specificity directed entirely to toxin.

The data presented here indicate that both anti-Forssman and anti-isophile serums possess at least two antibodies which vary in their hemolytic efficiency and in their sedimentation rate in a centrifugal field. The question raised is whether the demonstrated differences in physical property have a causal relationship with the differences in hemolytic efficiency.

EXPERIMENTAL PROCEDURE AND RESULTS

Forssman antiserums were obtained from rabbits after thrice weekly injections for 2 to 6 weeks with 1 ml/kg of suspensions of heated sheep red cell stromata containing 1.6×10^9 stromata/ml. Six serums were selected with varying ratios of hemolytic to combining activity.

Two isophile antiserums were obtained after a second course of 10% sheep red cell suspensions. Each course consisted of 6 injections of 1 ml/kg of the 10% red cells given over a 2-week period. A rest of 6 weeks separated the two courses. To remove Forssman antibody, both serums were absorbed 4 times with 3×10^{10} heated stromata per ml serum. Hemolytic titrations on the original serums and after the 1st and 4th absorptions are given in table 2.

After diluting 1:4 in saline, both the Forssman and isophile serums were centrifuged for 200 minutes in an average field of $105{,}000 \times g$ in a Spinco refrigerated centrifuge. The top, middle and bottom fractions were analyzed for hemolytic and combining activity. Results obtained with the Forssman antiserums are given in table 1, which lists the results of the measurement of hemolytic and combining units with one serum and the ratios of these two units with all six serums. The results obtained with the isophile antiserums are given in table 2.

TABLE 1.—*Ultracentrifugal separation of Forssman antibodies.*

Serum fraction	7			8	9	10	11	12
	H	C	H/C	H/C	H/C	H/C	H/C	H/C
Whole	1700	4.2	405	158	117	684*	113*	66*
Top	150	1.4	107	50	24	†	42	17
Middle	710	2.1	338	103	100	1130	106	53
Bottom	7100	9.4	755	291	264	1714	321	168

H/C is ratio of hemolytic to combining units.
* Since sufficient whole serum was not available in three cases, ratios were determined from the sum of titers obtained in the individual fractions.
† Combining units below level of accurate measurement

TABLE 2.—*Ultracentrifugal separation of isophile antibodies.*

Serum	Hemolytic titer before and after absorption with heated stromata		
	Original	After 1 absorption	After 4 absorptions
13	12,600	1,100	1,200
14	42,000	2,200	1,700
Centrifugal fraction	Hemolytic units/ml	Combining units/ml	H/C
13 Top	50	4.7	11
13 Middle	150	8.4	18
13 Bottom	600	25	24
14 Top	<10	1.1	<9
14 Middle	130	1.6	81
14 Bottom	1700	4.2	405

The hemolytic efficiency (ratio of hemolytic to combining capacity) increased progressively from the top to the bottom layer in all 8 serums. The average increase in ratio of the bottom fraction over the top was 10-fold. The greatest increase was 40-fold, the smallest 2-fold.

The total hemolytic and combining activity added to each centrifuge tube was compared with the sum of the amounts recovered from each fraction after centrifugation. The results obtained from the three serums on which this comparison was made are given in table 3. Although there was no signifi-

TABLE 3.—*Recovery of antibody after centrifugation.*

Serum no.	Hemolytic Units		Combining Units	
	Added	Recovered	Added	Recovered
7	21,250	22,050	52	41
8	10,500	10,250	66	54
9	7,000	8,600	60	49

cant loss in hemolytic units, there was an approximate 20% loss in combining units in each case.

DISCUSSION

The results presented indicate that both Forssman and isophile anti-sheep red cell serums contain two antibodies which differ in sedimentation rate and hemolytic efficiency. The evidence for this statement is as follows:

(1) The magnitude of the differences in hemolytic efficiency suggests a real difference in antibody rather than an experimental artefact. The difference in ratio of hemolytic to combining units between top and bottom fractions after centrifugation averaged 10-fold. Since both molecules are concentrated in the bottom fraction, their separation with a single centrifugation can be only partial. A complete separation of the two antibodies should give a much greater difference in hemolytic efficiency than the approximately 10-fold difference found in most of the tubes. There is a 100-fold difference between the ratio found in the bottom fraction of serum 10 (serum in table 1 with highest initial ratio) and the top fraction of serum 12 (serum with lowest initial ratio). A fractionation of anti-Forssman serums by starch block electrophoresis[15] yielded two distinct peaks for hemolytic and combining activity. The ratio of hemolytic to combining units at these two peaks differed by a factor of approximately 100.

(2) The change in ratio of hemolytic to combining units occurred in both directions from that found in the original serum. Some effect of centrifugation (e.g., aggregation) might alter the antibody in such a way as to change one unit of measurement more than the other. A single effect would not produce a change in ratio in both directions from the ratio of the original serum.

(3) A comparison of the sum of the hemolytic units present in each fraction with the total number added to the tube revealed no significant loss of hemolytic activity as a result of centrifugation. The separation of a two-component hemolytic system (aside from complement) should result in a net loss of total hemolytic activity.

(4) Passive transfer studies performed in normal rabbits with whole rabbit antisheep cell serum demonstrated marked differences in the disappearance curves of the Forssman hemolytic and combining activities.[16]

Granting the presence in rabbit antiserums to sheep red cells of two Forssman and two isophile antibodies, there are a number of possible explanations of the marked differences in hemolytic efficiency.

The hemolytic efficiency of the antibody may depend on the site on the red cell with which the antibody combines. Against this interpretation, however, is the fact that red cells may be hemolyzed by antibodies of different specificities[1] and by antibodies directed against antigens adsorbed[17] on or chem-

15. Stelos, P. and Talmage, D. W. 1956, Electrophoretic and ultracentrifugal studies of rabbit antiserum to sheep red blood cells and to boiled sheep RBC stromata. Fed. Proc. 15: 615.

16. Taliaferro, W. H. and Talmage, D. W. 1956, Antibodies in the rabbit with different rates of metabolic decay. J. Infect. Dis. 99: In press.

17. Fisher, S. and Keogh, E. V. 1950, Lysis by complement of erythrocytes which have absorbed a bacterial component and its antibody. Nature, 165: 248.

ically bound[18] to their surface. For this reason, it seems unlikely that there is a particular weak point on the surface of the red cell with which the antibody must combine before hemolysis occurs. The evidence that the two Forssman antibodies have a closely related specificity is based on the similarity of their absorption by varying numbers of red cells. This evidence has been presented and discussed in the preceding paper.[1] Even if it is assumed that minor differences in specificity exist between the two types of Forssman antibody, the sites on the red cell with which they combine must be arranged so closely that the absorption of one type of antibody blocks the absorption of the other.

If the hemolytic efficiency of an antibody molecule is not determined solely by the site on the red cell with which it combines, then it follows that some property of the antibody molecule itself is a factor in determining hemolytic efficiency. This determining factor might be the number or quality of the reactive sites, or it might be some general physicochemical property such as the size and shape of the molecule which would secondarily affect the union of antigen and antibody. Bowman, Mayer and Rapp[19] have suggested that hemolytic activity is related to the reversibility of the antigen-antibody complex. Present knowledge of antibody action is insufficient, however, to relate the size or valence of an antibody molecule to reversibility or to hemolytic efficiency.

A question remains as to whether the separation by physicochemical means of two Forssman antibodies of related specificity provides experimental evidence of a relationship between physicochemical property and hemolytic efficiency. If the antibodies of each type were completely homogeneous, there would be a fair probability of a chance correlation between two unrelated properties of the molecules. If, however, one considers the antibodies of both types to be inhomogeneous with respect to many functions, then the determinations of sedimentation rate and hemolytic efficiency represent a statistical average of all the molecules present. Under these circumstances, the probability becomes much smaller that the observed correlation was a matter of chance. The question might be resolved by an extension of the present method of study to a greater number of antigen-antibody systems. In this connection, consideration should be given to the fact that both the highly efficient hemolysin[15] and skin-sensitizing "reagin"[8,9] have been found between the β and γ globulin fractions of serum.

The hypothesis is not new that the general physicochemical properties of an antibody molecule can affect the biological manifestation of that antibody. It has been used to explain the skin-sensitizing ability of human "reagins."[20] However, the hypothesis is opposed to current concepts of the unity of antibodies[21] and will probably not be accepted unless a more definitive mechanism of antibody action is obtained in some biological system. The

18. Silverstein, A. M. and Maltaner, F. 1952, Hemolysis with complement of intact azo-erythrocytes sensitized with antisera homologous to the attached azo-grouping. J. Immunol. 69: 197–200.

19. Bowman, W. M., Mayer, M. M. and Rapp, H. J., 1951, Kinetic studies on immune hemolysis. II. The reversibility of red cell-antibody combination and the resultant transfer of antibody from cell to cell during hemolysis. J. Exper. Med. 94: 87–110.

20. Cooke, R. A. 1947, Allergy in Theory and Practice, Philadelphia, W. B. Saunders Co.

21. Bronfenbrenner, J. 1948, Hypersensitivity and immunity in the light of the "Unitarian" hypothesis. J. Allergy, 19: 71–89.

mechanism of hemolysis remains a promising approach to the solution of this problem.

SUMMARY

Analysis of top, middle and bottom fractions of 6 Forssman and 2 isophile antiserums separated by centrifugation for 200 minutes at 105,000 $\times g$ showed in every case a progressive increase from top to bottom of the hemolytic to combining ratio. The change in ratios from top to bottom fractions, which averaged tenfold, indicates the presence in these serums of at least 2 antibodies differing in sedimentation constant and in hemolytic efficiency.

The implication of these results to the unitarian concept of antibodies is discussed.

ASCITES AND HEPATOSPLENOMEGALY IN MICE ASSOCIATED WITH PROTOZOAN-LIKE CYTOPLASMIC STRUCTURES

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Since the report by Levaditi, Nicolau and Schoen[1] on the spontaneous occurrence of encephalitozoon infection in mice in France a number of articles have dealt with agents causing hepatosplenomegaly with an associated ascites in mice in American laboratories.[2–4] Perrin[2] drew attention to the presence in his affected mice of a protozoan similar morphologically to that described by Levaditi.[1] Jordan and Mirick[3] and Lakey et al[4] however, were unable to demonstrate such structures in the tissues of mice experimentally infected with their agents. Subsequently, in our laboratory during studies on far eastern hemorrhagic fever and on viral hepatitis, an enzootic disease of mice characterized by ascites, hepatosplenomegaly and presence of intracellular structures resembling protozoa was encountered. The present report deals with pathological, immunological and cultural studies on the etiologic agent of the disease and presents data which suggest that the new agent differs from *Encephalitozoon* which it resembles morphologically but is similar to, and probably identical with, the ascites-hepatitis agent described by Jordan and Mirick.[3]

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1. Levaditi, C., Nicolau, S. and Schoen, R. 1923, L'etiologie de l'encephalite. Compt. rend. Acad.d. sci. **177**: 985–988.
2. Perrin, T. L. 1943, Spontaneous and experimental encephalitozoon infection in laboratory animals. Arch. Path. **36**: 559–567.
3. Jordan, J. and Mirick, G. 1955, An infectious hepatitis of undetermined origin in mice. I. Description of the disease. J. Exper. Med. **102**: 601–616.
4. Lackey, M. D., Eichmann, P. L., and Havens W. P. 1953, Hepatitis in laboratory mice. J. Infect. Dis. **93**: 14–20.

MATERIALS AND METHODS

Isolation of strains of mouse ascitic agent (MAA).—Heart blood of a fatal case of serum hepatitis was inoculated intraperitoneally into a group of 8 to 10 g Bagg mice purchased from Mrs. Flora O'Grady. Suspensions of tissues of the inoculated mice were passed in series by intraperitoneal inoculation to other mice of the same strain at 21- to 28-day intervals. A transmissible illness characterized by ascites and hepatosplenomegaly associated with presence of protozoan-like bodies in the cytoplasm and nucleus of cells of the peritoneal exudate occurred in some of the 4th passage mice. In subsequent studies in which materials from patients with far eastern hemorrhagic fever were passed serially in Bagg mice and in which normal mouse tissues were similarly passaged, 12 strains of the mouse ascitic agent were recovered. These strains had the same general characteristics as those encountered in mice originally inoculated with hepatitis material.

Tissue culture.—Tube cultures of MB III cells were prepared as described by Owens et al,[5] except that roller tubes instead of "tumble" tubes were used. The fluid medium consisted of 50% horse serum, 40% Gey's balanced salt solution and 10% beef embryo extract; to this were added small amounts of penicillin and streptomycin (100 units and 20 μg/ml, respectively) to control bacterial contaminants. The cultures were maintained at 36 C. The nutrient fluid was changed at 3- to 4-day intervals by removing one-half of the contents of a tube (0.5 ml) and replacing it with an equal amount of new nutrient fluid. The infectious inoculum for the first passage in tissue culture consisted of 0.1 ml of undiluted ascitic fluid removed from a MAA infected mouse of the 30th passage on the 52nd day after experimental infection. Routinely, aliquots of materials used for subinoculation were

5. Owens, H. D., Gey, M. K. and Gey, G. O. 1954, Growth of cells in agitated fluid medium. Ann. N. Y. Acad. Sc. **58**: 1039–1055.

tested for infectivity in 1- to 3-day-old suckling mice and for presence of parasites by microscopic examination of stained cells.

Cross resistance tests.—Groups of male mice weighing 20 to 24 g were initially injected intraperitoneally with 0.2 to 0.5 ml of a suspension of mouse or chick embryo tissues infected with a known number of infectious units of MAA, the Walter Reed Army Institute of Research (WRAIR) strain of AHA (ascites-hepatitis agent of Jordan and Mirick[3]), and with 2 other agents producing ascitic disease in mice, i.e., the 113 strain of toxoplasma[6] and the Karp strain of *Rickettsiae tsutsugamushi*. Mice receiving the latter agent were given 250 mg of chloramphenicol in their drinking water daily for 21 days following injection to prevent deaths resulting from rickettsial infection.[7] Forty-one to 102 days after the initial injection all the animals were challenged on the same day by intraperitoneal inoculation of a suspension of mouse tissue or egg material containing the heterologous or homologous agent. In the challenge experiment the virulent RH strain of toxoplasma[8] was used instead of the avirulent 113 strain. At the same time, groups of normal mice were injected with the challenge material. Animals dead or with ascites at 21 days were recorded.

Histological procedures.—Tissue specimens for microscopic examination were fixed in Zenker's acetic acid solution. Paraffin sections were stained with hematoxylin and eosin or by the method of Giemsa or Goodpasture. Impression films of the surfaces of visceral organs of the inoculated animals were stained by the method of Macchiavello or Giemsa.

RESULTS

Behavior of MAA in mice.—Mice inoculated intraperitoneally with the MAA agent continue in apparent good health for 12 to 15 days, after which they become less active and develop abdominal swelling. Practically all sick mice begin to recover after 2 to 4 weeks of illness. However, mice killed 3 to 4 weeks after inoculation of infectious materials show enlarged, pulpy livers, splenomegaly and usually 5 to 10 ml of ascitic fluid. The most striking lesion is the ascites: in some infected mice as much as 30 ml of ascitic fluid is found at the height of the disease. Intracerebral injection of the MAA agent produced no obvious clinical illness or gross pathologic changes in mice.

Histologically the ascites is associated with considerable alteration of the mesothelial layer of the peritoneum.* Many of the normally flat mesothelial cells round up and in some areas they proliferate to the point of forming plaques or flat polypoid masses. Some of these cells in which protozoan-like structures are occasionally found (see fig. 1) are extruded into the peritoneal cavity and these plus a few small mononuclear cells together with a proteinaceous material form a coating on the peritoneal surface. Such changes are most frequently encountered in the parietal and mesenteric peritoneum rather than in that intimately associated with the abdominal visera.

The liver lesions are of three types. The first is a focal lesion scattered throughout the parenchyma which consists of a mononuclear infiltration often associated with a small group of necrotic liver cells. In addition, the periportal spaces and adjacent tissue are often infiltrated with masses of mononuclear cells and lymphocytes. Finally, there is a generalized thinning of the hepatic cords. Thus, the changes are similar to those described and illustrated in the report of Jordan and Mirick.[3]

The bronchial, mesenteric and subcutaneous lymph nodes show evidence of chronic lymphadenitis: there is hyper-

6. Jacobs, L., Melton, M. L. and Cook, M. K. 1953, Experimental toxoplasmosis in pigeons. Exper. Parasitol. **2**: 403–416.
7. Smadel, J. E., Jackson, E. B. and Crusie, A. B. 1949, Chloromycetin in experimental rickettsial infections. J. *Immunol.* **62**: 49–65.
8. Sabin, A. B. 1941, Toxoplasmic encephalitis in children. J. A. M. A. **116**: 801–807.

* The pathological studies reported here were done jointly with Lee Wattenberg, 1st Lt., MC USA.

FIG. 1.—A cyst-like structure containing parasites in a cell of the parietal peritoneum of a mouse sacrificed 21 days after experimental infection. Giemsa stain. ×2000.

FIG. 2.—A group of MB cells from tissue culture 28 days after exposure to mouse ascitic fluid infected with MAA. Masses of MAA are seen within the cytoplasm of cells. Giemsa stain. ×2000.

plasia of reticuloendothelial cells and many of these, along with other mononuclear cells, are seen within the sinuses. The sinuses themselves are considerably dilated. The spleen, aside from hyperplasia, appears essentially normal as does the brain,† heart, lungs, small bowel and kidneys.

Behavior of MAA in MB cell tissue culture.—Inoculation of MAA obtained from ascitic fluid of an infected mouse into cultures of MB III cells produced visible parasitization of culture cells by the 21st day, see fig. 2. Infected cells when stained by the method of Giemsa have intracytoplasmic and intranuclear bodies which take a blue strain surrounded by a clear zone; when stained by the method of Macchiavello such structures are red. The infected cells do not undergo immediate degeneration as a result of infection, but appear to increase in size as the parasite multiplies within it. This is followed by disruption of the infected cell and freeing of the parasites into the medium. Inoculation of infected MB III cells into susceptible mice resulted in disease similar in all respects to that induced in mice by injection of suspensions of infected mouse tissues.

Attempt to cultivate MAA in other animals and embryonated eggs.—Suspensions of mouse tissues containing 10 to 100 mouse infective doses of MAA were inoculated into 250 to 300 g guinea pigs, young adult rabbits, hamsters, rhesus monkeys and rats by the intracerebral and intraperitoneal routes. With the exception of moderate ascites which was present in 1 of 2 albino rats inoculated with early mouse passage material (attempts to repeat these results with late mouse passage materials failed), none of the inoculated animals

† In 1 of 15 brains examined a slight focal mononuclear infiltration was present in the thalamus. This single lesion may have been due to a concomitant infection with an agent other than MAA. If it were indeed specifically associated with the MAA infection then involvement of the central nervous system appears to be a relatively uncommon feature of the experimental disease induced by intraperitoneal inoculation of the agent.

developed signs of illness and MAA was not found in Macchiavello stained impression films of the visceral tissues or brains of the inoculated animals when sacrificed 21 to 30 days after inoculation.

Nine-day-old embryonated eggs inoculated by the yolk sac, amnionic or allantoic cavity routes with infectious mouse tissues showed no evidence of infection and embryonic tissues and fluids harvested from some of the eggs 3 to 5 days after inoculation did not contain demonstrable MAA. Furthermore, the tissue of such embryos were not infectious for mice.

Attempt to cultivate MAA on artificial mediums.—Bacterial cultures of MAA infected mouse tissues made on blood agar slants and thioglycolate from each suspension used for passage gave negative results except for an occasional bacterial contaminant. In addition, the MAA agent failed to grow in special mediums capable of supporting the growth of histoplasma and other fungi, the pleuropneumonia and leptospires. From the tissues of one mouse *Leptospira ballum* was recovered. None of these contaminants was capable of producing the ascitic disease in mice.

Cross resistance tests in mice with MAA, AHA, toxoplasma and scrub typhus.—The results of cross resistance tests performed in mice convalescent from MAA and toxoplasma are shown in table 1, test no. 1. It is seen that mice injected with each agent developed immunity to challenge with the homologous but not the heterologous agent. In another cross resistance test (table 1, test no. 2) efforts were made to determine whether the disease in our mice and that described by Jordan and Mirick[3] have a common etiology. The agent of the latter disease was received at the WRAIR in March 1952 in the 15th mouse passage and has been maintained by serial passage in Bagg mice. After protozoan-like structures were found in tissues of mice infected with MAA an intensive search was begun at the 27th passage level for similar bodies in stained impression films of tissues of mice infected with AHA. These were found in mice of the 28th passage, but it is difficult to assess the significance of their presence since it is established that apparently healthy Bagg mice harbor latent MAA infections. Nevertheless, cross resistance tests were performed in mice with MAA and the WRAIR strain of AHA. It is apparent from the data presented that MAA and AHA convalescent mice were resistant to reinfection with the homologous agent as

TABLE 1.—*Cross resistance tests in mice between MAA, AHA and toxoplasma.*

Test no.	Challenged with:	Convalescent from:	Number mice dead or with ascites 21 days after challenge with dilutions of:								
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1	MAA	Controls	5/5*	3/6	0/6						
		MAA	0/4								
		Toxoplasma (113)	5/5								
	Toxoplasma (RH strain)	Controls				6/6	6/6	3/6	0/6		
		MAA			5/5	5/5					
		Toxoplasma (113)			6/6	5/5	0/6				
2	MAA	Controls	6/6	6/6	3/6	0/6					
		MAA	1/9								
		AHA	0/9								
		Scrub tryphus (Karp strain)	16/16								
	AHA	Controls	8/8	8/8	2/7	1/8					
		MAA	0/36								
		AHA	0/29								

5/5 = Number mice with ascitic disease or dead at 21 days/number mice inoculated.

well as heterologous agent. Further, mice convalescent from unrelated ascitic diseases (scrub typhus and toxoplasma) were fully susceptible to infection by MAA. It may be mentioned here that encephalitozoa were not included in these cross resistance tests since overt disease was produced in only 10% to 15% of the mice inoculated with the strains of this parasite available to us.

It is of interest, in view of the homologous immunity demonstrated in mice, that convalescent serums from recovered mice do not have a neutralizing effect on the MAA agent when tested in mice or in tissue culture. Further, the immunity in mice to MAA and AHA is short-lived. To illustrate the latter point, 3 groups of mice, one group inoculated with AHA 68 days before challenge and the other groups with MAA 58 and 69 days previously, resisted challenge with 4000 50% infectious doses of AHA. In contrast, certain mice challenged in the same test which had been inoculated originally 90 and 110 days earlier with MAA and AHA, respectively,were not resistant: 4 of the 6 AHA convalescent mice and 2 of the 4 MAA convalescent mice developed ascitic disease at the regular time following challenge.

The effect of physical agents on MAA.—MAA survives exposure to 56 C for 30 minutes and to −70 C for prolonged periods, but is inactivated by lyophilization. It is completely sedimented by centrifugation at 2500 r.p.m. for 1 hour and is held back by a Seitz EK filter pad. These results suggest that the etiologic agent is rather large and add weight to the opinion that the intracellular protozoan-like structure is or contains the infectious agent.

The effect of antimicrobial agents on MAA infection.—Several antimicrobial agents were tested in separate groups of mice which were given the initial dose of drug 2 to 24 hours after experimental infection with the MAA agent. Penicillin (500 units) and streptomycin (100 mg) were injected intraperitoneally daily for 10 days. Chloramphenicol was added to the drinking water, each mouse received approximately 250 mg each day for 21 days. Daraprim (5 mg) and sulfadiazine (5 mg) were administered intraperitoneally daily for 16 days. None of the drugs tested exerted any apparent influence on the course of MAA infection in mice.

DISCUSSION

The etiologic agent of an enzootic disease characterized by ascites and hepatosplenomegaly and associated with intracellular bodies resembling protozoa encountered in mice during isolation studies on viral hepatitis appears to be different from a number of well defined agents inducing similar diseases in mice and may be a hitherto undescribed protozoan. The disease resulting from infection with the new agent differs from that ordinarily resulting from infection with *Encephalitozoon*. The most constant and characteristic lesion in encephalitozoon infected mice is a meningoencephalitis.[2] This lesion was not present in any of an appreciable number of mice infected with MAA, although focal monocytic infiltration of the thalamus was seen in 1 of 15 brains examined. Furthermore, the grapelike clusters of parasites commonly present in the kidneys of mice infected with *Encephalitozoon* are not seen in kidneys of mice infected with MAA. A determination of the possible taxonomic relationship of MAA to *Encephalitozoon* might be assisted by the development of suitable serologic tests. Perhaps the successful propagation in tissue culture of MAA may provide an effective tool in establishing the true taxonomic and immunologic relationships of some

members of the ill-defined group of organisms now lumped together by some authors into the subclass Acnidosporidia of the class Sporozoa.

The disease in our mice and two other ascitic diseases encountered in mice by Jordan and Mirick[3] and by Lackey[4] are similar in many respects. Each has an incubation period of 2 to 4 weeks, low mortality (3%), splenomegaly and hepatic lesions consisting of necrosis of parenchymatous cells and mononuclear infiltrations situated perivascularly and focally throughout the parenchyma. Further, our agent and that of Jordan and Mirick share many characteristics such as pathogenicity for mice with essential avirulence for other species of animals, infectivity of organ suspensions at dilutions 10^{-2} to 10^{-3}, resistance to temperatures of -70 C for prolonged periods and 56 C for 30 minutes, resistance to common antibiotics and sulfathiazine, and inability to evoke a demonstrable antibody response in mice. Both agents are sedimented by centrifugation at 2500 r.p.m. for 1 hour and are held back by a Seitz EK filter. The disease in our mice, however, is consistently associated with the intracellular protozoan-like structures. Such cytoplasmic structures have not been described in the ascitic diseases studied by Jordan and Mirick or by Lackey et al. Since the parasites associated with MAA infection were seldom numerous in the stained films of infected organs and often were found only after considerable search, it is possible that this is the same ascitic disease encountered in mice in other laboratories engaged in studies on human hepatitis.

Finally, transmissible disease indistinguishable from that asociated with MAA was encountered in mice during passage of tissues from "normal" Bagg mice and on other occasions in passage mice employed in search for the agent of far eastern hemorrhagic fever in human and animal materials. It is apparent, therefore, that MAA is enzootic in certain lines of laboratory mice.

SUMMARY

An enzootic disease of mice characterized by ascites, hepatosplenomegaly and presence of intracellular structures resembling protozoa is reported. The etiologic agent of this disease appears to be different from a number of well-defined agents causing similar diseases in mice and may be a previously undescribed protozoan. Certain of the pathogenic, cultural and physical properties of the new agent are presented.

RELATIONSHIP OF THE IN VIVO FORM OF *COCCIDIOIDES IMMITIS* TO VIRULENCE

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A striking characteristic of the fungi which cause deep-seated infections is their nonmycelial form in the tissues, while in the usual laboratory cultures they retain a mold-like appearance. The nonmycelial form found in the infected host seems particularly well suited for growth in vivo. Duncan[1] stated, "with the pathogenic fungi, therefore, virulence (interpreted in the restricted sence of infectivity and invasive power) would seem largely a matter of adaptability, morphological and physiological, to parasitic life in animal tissues." Norden[2] reiterated the apparent dependence of virulence on this "adaptability." *Rhinosporidum seeberi*, a human pathogen which in infected tissues resembles the tissue phase of the fungus *Coccidioides immitis*, may be an extreme example of adaptation, possibly irreversible, since it has not been cultured away from the tissues even in a moldlike form.[3] Baker[4] demonstrated that if mice were infected with equal weights of either the filamentous form or yeast cells of *Blastomyces dermatitidis*, the resultant lesions contained exclusively the yeast cell form. Similar examples might be cited for other fungi, e.g., *Histoplasma capsulatum*. Mackinnon[5] found that mutants of *Candida albicans* which grew only in a filamentous form were nonpathogenic for rabbits, while their parent strains which produced yeast cells retained their pathogencity.

Similarly, *C. immitis* invariably exists in its sporangial (spherule) or nonfilamentous form in human and animal lesions. In the recent discussions by Puckett[6] and Fiese et al,[7] of the occasional existence of the mycelial form of this fungus in the walls of pulmonary cavities, they suggest that the wall of a cavity may be considered as a focus exterior to the tissues proper. Hence, it seems generally true that the filamentous form of *C. immitis* will not exist in the tissues.

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The opinions contained in this report are not to be construed as reflecting the views of the Navy Department or the Naval Services at large. (Article 1252, U. S. Navy Regulations, 1948). Reproduction in whole or in part is permitted for any purpose of the United States Government.

1. Duncan, J. T. 1947, Virulence of fungi in animals. J. Gen. Microbiol. **1**: iv.
2. Norden, A. 1951, Sporotrichosis, clinical and laboratory features and a serologic study in animals and humans. Acta. path. et microbiol. Scandinav., Supp. 89.
3. Conant, N. F., Martin, D. S., Smith, D. T., Baker, R. D. and Callaway, J. L. 1954, Manual of Clinical Mycology, ed. 2, Philadelphia, W. B. Saunders Co.
4. Baker, R. D. 1938, Comparison of infection of mice by mycelial and yeast forms of *Blastomyces dermatitidis*. J. Infect. Dis. **63**: 324–329.
5. Mackinnon, J. E. 1940. Dissociation in *Candida albicans*. J. Infect. Dis. **66**: 59–77.
6. Puckett, T. F. 1954, Hyphae of *Coccidioides immitis* in tissues of the human host. Am. Rev. Tuberc. **70**: 320–327.
7. Fiese, T. F., Cheu, S. and Sorensen, R. H. 1955, Mycelial forms of *Coccidioides immitis* in sputum and tissues of the human host. Ann. Int. Med. **43**: 255–270.

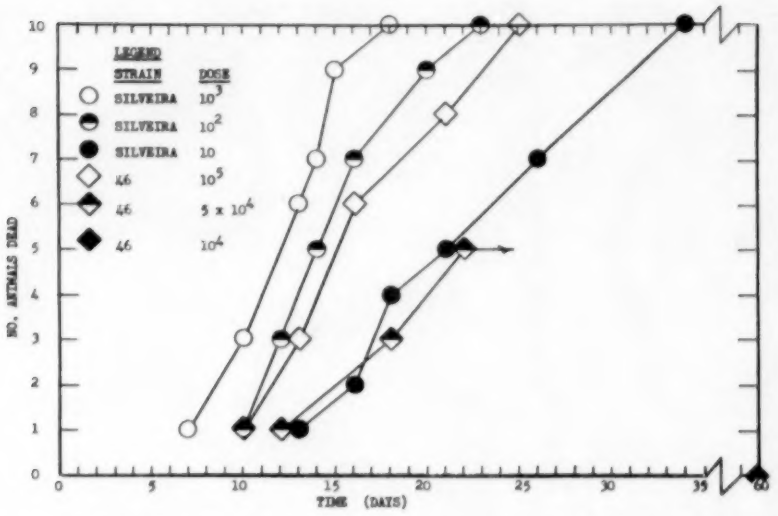

FIG. 1.—Comparative virulence of arthrospores of two strains of *C. immitis* in mice infected intraperitoneally.

The present report deals with experiments on two strains of *C. immitis* which differ markedly in virulence for mice. We have attempted to determine what importance could be attached to the attainment of the in vivo form in the virulence of this fungus.

MATERIAL AND METHODS

Strains tested.—The strains of *C. immitis* used had been obtained from human infections. Strain 46, the relatively avirulent strain, was isolated in 1933 from a patient with a disseminated infection who subsequently died of coccidioidal meningitis. Its low virulence for the mouse was noted by one of us in 1946. The second strain used, strain Silveira, was isolated in 1951 from another patient with a severe coccidioidal infection who, however, recovered. Friedman et al[8] have mentioned the difference in virulence of the arthrospores of these two strains. In figure 1 we have shown the extent of this difference in virulence. While as few as 10 arthrospores of strain Silveira were found to cause lethal infections by the intraperitoneal route in mice, strain 46 was only lethal in doses of 10^4 or more arthrospores.

Source of in vivo form.—Male white mice 20 to 25 g in weight were inoculated intraperitoneally with approximately 10^6 arthrospores of each strain, harvested from 4- to 6-week-old cultures on 2% glucose—1% yeast extract agar. At 11 days following infection, 5 mice, of each group most of which were acutely ill, or dying, were sacrificed and their lungs removed. The lungs, heavily studded with 1 to 2 mm nodules containing many coccidioidal spherules, were ground in a sterile mortar to a fine paste which was suspended in sterile physiological saline. Microscopic examination revealed that the suspensions contained virtually no intact mature spherules, i.e., the spherules had been ruptured, releasing their endospores (fig. 2). Serial 10-fold dilutions of these endospore suspensions were counted by pour plate technique using the agar medium of Roessler et al.[9] The plates were incubated at 34 C for 48 hours, at which time colony counts were made. The suspensions, which had been maintained at 3 C during the incubation of the plates, were adjusted to the desired concentration of

8. Friedman, L., Smith, C. E., Roessler, W. G. and Berman, R. J. 1955. The virulence and infectivity of *Coccidioides immitis* grown on solid and liquid (submerged) media. Bact. Proc. p. 107.

9. Roessler, W. G., Herbst, E. J., McCullough, W. G., Mills R. C. and Brewer, C. R. 1946, Studies with *Coccidioides immitis* I. Submerged growth in liquid mediums. J. Infect. Dis. **79:** 12–22.

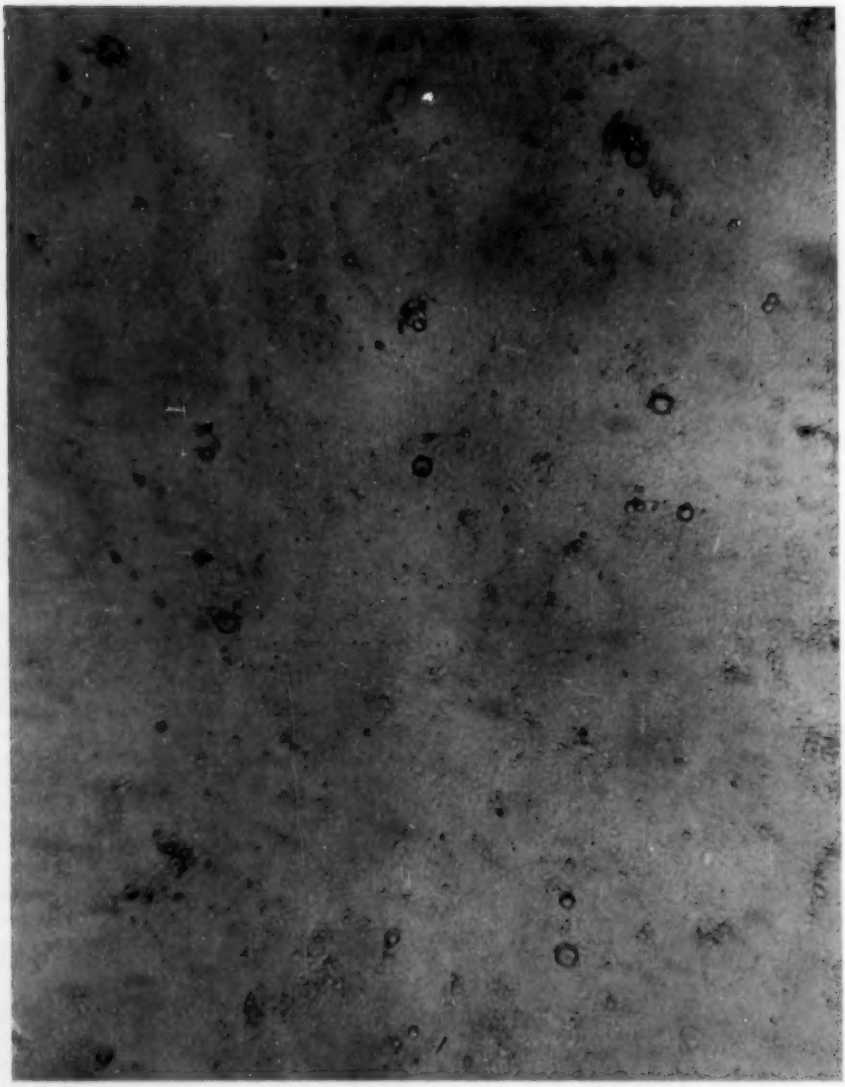

FIG. 2.—Endospores freed from spherules by trituration of infected mouse lung. ×800.

endospores. Male white mice 20 to 25 g in weight were inoculated intraperitoneally with 0.5 ml of the appropriate dilution and the endospore dosage rechecked by pour plate count of the inoculum.

For purposes of comparison, a control group of mice was infected in a similar manner with a suspension of arthrospores of strain Silveira, the spores having been obtained from the glucose-yeast extract agar as previously described. Noteworthy was the appearance of colonies within 24 hours in the pour plates inoculated with endospores while those with arthrospores required incubation for 72 to 96 hours before they were visible. Therefore, arthrospore counts were begun 4 days prior to inoculation into mice, while endospore suspensions from macerated tissue were prepared and counted 2 days before the date of inoculation.

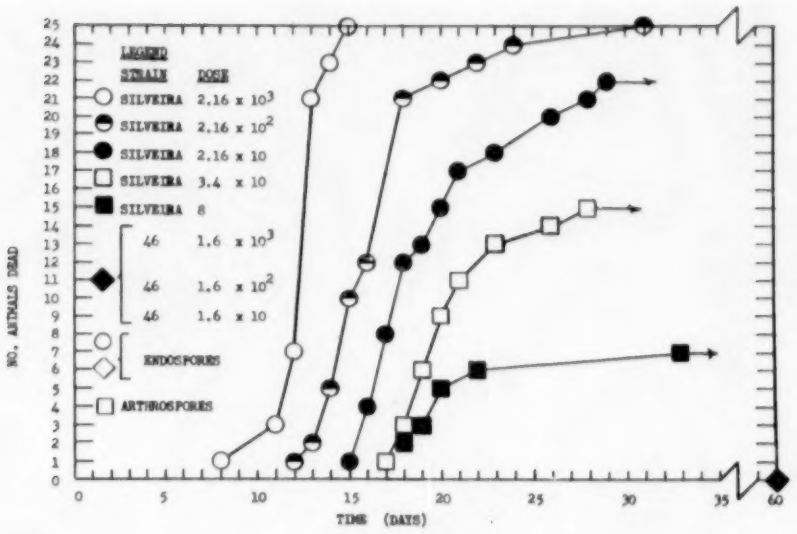

FIG. 3.—Comparative virulence of the in vivo form (endospores) of two strains of *C. immitis*.

Germination rate of endospores and arthrospores.—In view of the earlier appearance of visible colonies from endospores, hanging drop preparations of both spore forms were examined periodically for evidence of germination. Spores were suspended in glucose-yeast extract broth and placed in van Tieghem cells. A modification of the latter which ensured abundant aeration was made by grinding a groove about 2×2 mm in the vertical portion of the cell on the end distal to the coverslip. The hanging drops were incubated at 34 C in Petri dishes containing sterile water to prevent drying.

RESULTS

Figure 3 and table 1 summarize the results of the experiments. The two experiments recorded in table 1 indicate the lethality of the Silveira arthrospores in accordance with past experience[8] although in the experiment graphed in figure 3 the mice injected with Silveira arthrospores manifested unusual resistance. In the three experiments vari-

TABLE 1.—*Comparative virulence of the in vivo form (endospores) of two strains of* C. immitis *in white mice.*

Exp.	Strain	Form	Dose	Number of mice	Deaths on day:												Dead/total
					14	15	16	17	18	19	20	21	22	23	24	25–60	
1	Silveira	Endospores	150	10	1	0	1	1	0	2	1	0	1	0	1	2	10/10
	46	Endospores	800	10	0	0	0	0	0	0	0	0	0	0	0	0	0/10
	46	Endospores	80	10	0	0	0	0	0	0	0	0	0	0	0	0	0/10
	46	Endospores	8	10	0	0	0	0	0	0	0	0	0	0	0	0	0/10
	Silveira	Arthrospores	105	10	2	0	4	0	2	2							10/10
					Deaths on day:												
					14	16	17	18	22	23	28	30	35	36–60			
2	Silveira	Endospores	1400	10	0	2	4	0	0	0	0	0	1	1			8/10
	Silveira	Endospores	140	10	0	0	3	0	1	0	0	0	0	0			4/10
	Silveira	Endospores	14	10	0	0	1	0	0	1	0	0	1	0			3/10
	46	Endospores	7000	10	0	0	0	0	0	0	0	0	0	0			0/10
	46	Endospores	175	10	0	0	0	0	0	0	0	0	0	0			0/10
	46	Endospores	17	10	0	0	0	0	0	0	0	0	0	0			0/10
	Silveira	Anthrospores	47	10	0	0	1	4	1	1	1	1	1				10/10

FIG. 4.—Germination of endospores (strain Silveira) after 5 hours at 34 C. Hanging drop preparation. ×1200.

able numbers of arthrospores and endospores of strain Silveira were used, but the virulence of the arthrospores and endospores appeared comparable. The gross pathologic picture of animals infected with either form of strain Silveira was similar, exhibiting extensive lesions on peritoneal surfaces, spleen and liver as well as throughout the lungs. On the other hand, the lesions visible in animals infected with endospores of strain 46 were limited to small areas in the omentum and diaphragm.

The endospores of strain 46 had the

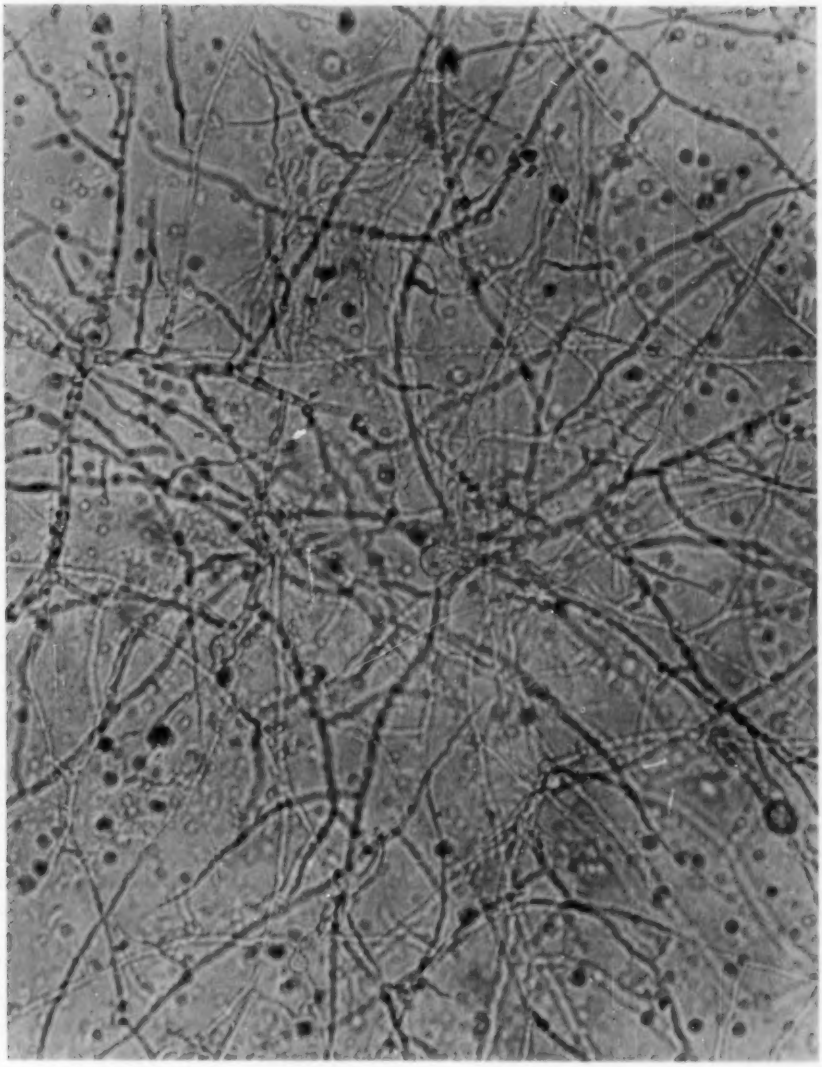

FIG. 5.—Germination of endospores (strain Silveira) after 16 hours at 34 C. Hanging drop preparation. ×1200.

same relative avirulence as the previously studied arthrospores. Thus, as figure 3 reveals, 1600 endospores of strain 46 failed to kill any mice in 40 days, while 21 endospores of strain Silveira killed 23 of 25 mice in 37 days. As indicated in table 1, even 7000 endospores of strain 46 caused no deaths in 60 days.

Observation of germination of the endospores revealed that in both strains short hyphal tubes developed after 4 to 5 hours at 34 C. This is shown in figure 4. In the arthrospores such germ tubes

appeared only after 13 to 15 hours incubation. By this time the incubated endospores had formed the tangled network of hyphae shown in figure 5. Moreover, endospores at the periphery of ordinary wet mounts germinated more rapidly than did those in the center, probably due to more favorable aerobic conditions.

DISCUSSION

Whereas the existence in the tissues of the nonfilamentous form of the pathogenic fungi may indicate a form adapted to the rigors of a parasitic modus vivendi, the present studies indicate that mere attainment of the in vivo morphology does not guarantee virulence (progressive disease). Some factor other than capacity to transform from arthrospore to spherule is responsible for the lack of virulence in strain 46. Comparison of the proximate chemical composition of the mycelia has not revealed a striking difference between strain 46 and the virulent strain Silveira.[10]

The present experiments have not shown a significant difference in virulence between the in vivo endospores and arthrospores. While Tager and Liebow[11] reported a more "acute mortality" in mice intranasally infected with spherules and endospores of *C. immitis* than those receiving the mycelial form, they felt that this might be due to heavier dosage of the tissue phase. Baker and Braude[12] noted that spherules injected into irradiated neutropenic mice induced more rapid formation of lesions than mycelia. It was inferred that spherule development depended on the aggregation of leucocytes about mycelia, and the absence of leucocytes in neutropenic mice delayed lesion formation. This appears to support the idea advanced by Scherr[13] that if evolution to the nonfilamentous or yeast cell form of the fungi can be prevented, perhaps the infection could be limited.

It was of interest to note that Newcomer et al[14] reported that only 3 of 18 mice infected with 84,000 spherules were dead in 15 days, while in our experiment 2160 endospores of strain Silveira killed all 25 mice, and 216 endospores killed 15 of 25 mice in the same time period. A more chronic type of infection appeared to have been induced by the strain used by Newcomer and his coworkers since deaths continued to occur at least through 67 days. Unfortunately, it is not possible to assess the virulence of the in vitro form of their strain, no counts of this inoculum being given.

There are many analogies between tuberculous and coccidioidal infections.[15] One striking difference, however, is the frequent bronchogenic spread of tuberculosis from an open pulmonary cavity with its resultant poor prognosis in contrast to the usually benign nature of the coccidioidal cavity.[16] While the

10. Pappagianis, D. 1955. Unpublished data.
11. Tager, M. and Liebow, A. 1942, Intranasal and intraperitoneal infection of the mouse with *Coccidioides immitis*. Yale J. Biol. & Med. **15**: 41–59.
12. Baker, O. and Braude, A. I. 1955, Abstract, 9th Annual Meeting So. Sect. Am. Fed. Clin. Res.
13. Scherr, G. 1952, Studies of the dimorphism mechanism in *Saccharomyces cerevisiae*. Mycopath. Mycol. Appl. **6**: 182–230.
14. Newcomer, V. D., Wright, E. T., Tarbet, J. E., Winer, L. H. and Sternberg, T. H. 1953, The effects of cortisone on experimental coccidioidomycosis. J. Inv. Dermat. **20**: 315–327.
15. Smith, C. E. 1942, Parallelism of coccidioidal and tuberculous infections. Radiology, **38**: 643–648.
16. Smith, C. E., Beard, R. R. and Saito, M. T. 1948, Pathogenesis of coccidioidomycosis with special reference to pulmonary cavitation. Ann. Int. Med. **29**: 623–655.

favorable status of coccidioidal cavity probably is mainly attributable to the immunity of the host, there may also be a mycologic aspect of significance. As has been shown, the endospores germinate very rapidly outside the tissues. Spherules and endospores gaining access to the aerobic environment in an open cavity or bronchial passage would germinate rapidly. As young hyphae they may not be successfully phagocytosed and transported back into the host's tissues because of relatively large dimensions, but instead be more readily removed from the lungs. Hence, they would not provide new foci of infection as seen in the bronchogenic spread of tuberculosis. Those hyphae which develop from spherules and remain in the fibrotic walls of coccidioidal cavities[6,7] are probably quite firmly fixed, and the aerobic and fibrotic environment may prevent conversion to spherules.

SUMMARY

Two strains of *Coccidioides immitis* which were known to differ markedly in virulence for mice in the arthrospore stage, were shown to differ also when in the endospore (in vivo) form. It was concluded that attainment of the spherule form per se would not affect virulence. Virulence of arthrospores and endospores appeared to be comparable by the intraperitoneal route. Endospores appeared to be very active metabolically, germinating some 10 hours earlier than arthrospores under similar aerobic conditions.

GENERAL INDEX, VOLUME 98, NO. 1, 2, AND 3 (1956)

Adler, Louise T.: The effect of thyroxin on respiration and virus growth in chick embryo tissue cultures, 239
Ascites, and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures, 306
Aulisio, C. G.: Serological evidence of toxoplasmosis in animals, 52

Bartonella, Concerning the mechanism of bartonella anemia in splenectomized rats, 113
Baumeister, Jacqueline: The preparation of poliomyelitis complement-fixing antigens from infected tissue culture fluids, 27
Benavides V., Lázaro: Pathological physiology of salmonellosis.
II. Plasma cholinesterase in infections due to Enterobacteriaceae, 1
Berendt, Richard F.: The effect of nasal secretions upon human serum, 223
Bergman, E. N.: Experimental canine leptospirosis.
I. *Leptospira icterohemorrhagiae* infections in immature dogs, 249
II. Renal function studies, 260
Bernkopf, Hans: Isolation of six strains of ornithosis virus from children with infections of the respiratory tract, 45
Berry, L. Joe: Susceptibility to infection as influenced by acclimatization to altitude and Krebs cycle inhibitors and intermediates, 21
Dynamics of bacterial infections in mice under conditions known to alter survival time, 198
Blount, R. E.: Ascites and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures, 306
Burgdorfer, Willy: Experimental studies on argasid ticks as possible vectors of tularemia, 67

Candida, Enhancement of candida infection: differential distribution of renal lesions in mice treated with aureomycin, 150
Carillo, Julio: Pathological physiology of salmonellosis.
II. Plasma cholinesterase in infections due to Enterobacteriaceae, 1
Cavanaugh, D. C.: Studies on *Pasteurella pestis* in various flea species.
I. Infection of flea species with avirulent strains of *Past. pestis*, 103
II. Simplified method for the experimental infection of fleas, 107
Chamberlain, R. W.: Transmission studies with encephalomyocarditis virus, 98
Chun, Doki: Demonstration of *Shigella flexneri* antigens by means of hemagglutination test, 82
Coccidioides immitis, Relationship of the in vivo form of, to virulence, 311
Coker, Coit M.: Some effects of cortisone in mice with acquired immunity to *Trichinella spiralis*, 39
Cellular factors in acquired immunity to *Trichinella spiralis*, as indicated by cortisone treatment of mice, 187
Colón, Julio I.: The effect of penicillin on multiplying and nonmultiplying populations of sensitive and resistant strains of feline pneumonitis virus, 229
Combining capacity, The blocking of labeled antibody uptake as a measure of the relative combining capacity of anti-red cell serums, 277
The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums, 293
Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation, 300
Cortisone, Some effects of, in mice with acquired immunity to *Trichinella spiralis*, 39
Criollos T., Oscar: Pathological physiology of salmonellosis.
II. Plasma cholinesterase in infections due to Enterobacteriaceae, 1
Cryptococcus, Studies on the biology of, 208

Dengue, Cultivation of mouse-adapted dengue virus (type 1) in rhesus monkey tissue culture, 88
De Ropp, Madeleine K.: Dynamics of bacterial infections in mice under conditions known to alter survival time, 198
Dicke, R. J.: Potential insect vectors of vesicular stomatitis in Wisconsin, 121
Donaldson, A. W.: The flocculation test with a purified antigen in the diagnosis of trichinosis in humans, 172

Eaton, Monroe D.: The effect of thyroxin on respiration and virus growth in chick embryo tissue cultures, 239
Eidson, M. E.: Experimental rabies infection in insectivorous bats, 10
Eiseman, Gilbert: Concerning the mechanism of bartonella anemia in splenectomized rats, 113
Encephalomyelitis, The comparative pathology of Venezuelan equine encephalomyelitis, 55
Encephalomyocarditis, Transmission studies with, 98
Effects of animal viruses on host cell metabolism, 270
Ephrati-Elizur, Erela: Isolation of six strains of ornithosis virus from children with infections of the respiratory tract, 45
Evans, Charles A.: Cultivation of mouse-adapted dengue virus (type 1) in rhesus monkey tissue culture, 88

Fair, Marjory H.: Dynamics of bacterial infections in mice under conditions known to alter survival time, 198
Ferris, D. H.: Potential insect vectors of vesicular stomatitis in Wisconsin, 121
Foley, G. E.: Enhancement of candida infection: differential distribution of renal lesions in mice treated with aureomycin, 150
Studies on the biology of *Cryptococcus.*
VI. Amino acid composition of the somatic proteins of *C. neoformans*, 208
Foot-and-mouth disease, Detection of complement fixing antibodies against, in cattle serum, 266
Freter, Gloria G.: The blocking of labeled antibody uptake as a measure of the relative combining capacity of anti-red cell serums, 277
The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums, 293
Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation, 300
Fusospirochete, The pathogenic components of an experimental fusospirochetal infection, 15

γ globulin, Antibody and γ globulin formation in vitro in hemopoietic organs, 157
Gleiser, Chester A.: Experimental canine leptospirosis.
I. *Leptospira icterohemorrhagiae* infections in immature dogs, 249
II. Renal function studies, 260
Grainger, R. M.: The pathogenic components of an experimental fusospirochetal infection, 15

Hanson, R. P.: Potential insect vectors of vesicular stomatitis in Wisconsin, 121
Hemolysin, The restoration of hemolysin-forming capacity in X-irradiated rabbits by tissue and yeast preparations, 75
Hiatt, C. W.: Experimental canine leptospirosis.
I. *Leptospira icterohemorrhagiae* infections in immature dogs, 249
II. Renal function studies, 260
Hotta, Susumu: Cultivation of mouse-adapted dengue virus (type 1) in rhesus monkey tissue culture, 88
Hughes, Francis W.: Effects of hypoxia on rats infected by *Trypanosoma lewisi*, 127

Innes, J. R. M.: Epizootic *Salmonella enteritidis* infection causing septic pulmonary phlebothrombosis in hamsters, 133

Jaroslow, Bernard N.: The restoration of hemolysin-forming capacity in X-irradiated rabbits by tissue and yeast preparations, 75

Katz, Edward: The activity of tetracycline on feline pneumonitis virus infection of chick embryos, 177
Keuning, F. J.: Antibody and γ globulin formation in vitro in hemopoietic organs, 157
Kissling, R. E.: Experimental rabies infection in insectivorous bats, 10
Transmission studies with encephalomyocarditis virus, 98
Knoll, M. L.: The pathogenic components of an experimental fusospirochetal infection, 15
Kobayashi, G. S.: Relationship of the in vivo form of *Coccidioides immitis* to virulence, 312
Krebs cycle, Susceptibility to infection as influenced by acclimatization to altitude and Krebs cycle inhibitors and intermediates, 21
Krishnamurty, D.: Paratido-stomatitis in calves, 142
Kumate, Jesús: Pathological physiology of salmonellosis.
II. Plasma cholinesterase in infections due to Enterobacteriaceae, 1

Leptospirosis, Leptospirosis canicolaris in pigs and its probable transfer to human beings, 33
Experimental canine leptospirosis, 249, 260
Levy, Hilton B.: Effects of animal viruses on host cell metabolism.
I. Effects of encephalomyocarditis virus on nucleic acids and phosphorus fractions of Ehrlich ascites tumor cells, 270
Low, Donald G.: Experimental canine leptospirosis.
I. *Leptospira icterohemorrhagiae* infections in immature dogs, 249
II. Renal function studies, 260

Macdonald, J. B.: The pathogenic components of an experimental fusospirochetal infection, 15
Madiener, E. M.: The pathogenic components of an experimental fusospirochetal infection, 15
McCown, J. M.: Serological evidence of toxoplasmosis in animals, 52
Ascites and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures, 306
Miller, C. Arden: The preparation of poliomyelitis complement-fixing antigens from infected tissue culture fluids, 27
Morris, J. A.: Serological evidence of toxoplasmosis in animals, 52
Ascites and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures, 306
Moulder, James W.: The effect of penicillin on multiplying and nonmultiplying populations of sensitive and resistant strains of feline pneumonitis virus, 229

Nasal secretions, The effect of, upon human serum, 223
Nordberg, B. K.: Detection of complement fixing antibodies against foot-and-mouth disease in cattle serum, 266
Norman, Lois: The flocculation test with a purified antigen in the diagnosis of trichinosis in humans, 172
Nungester, Walter J.: The effect of nasal secretions upon human serum, 223

Ornithosis, Isolation of six strains of ornithosis virus from children with infections of the respiratory tract, 45
Owen, Cora Rust: Experimental studies on argasid ticks as possible vectors of tularemia, 67

Pande, P. G.: Paratido-stomatitis in calves, 142
Pappagianis, D.: Relationship of the in vivo form of *Coccidioides immitis* to virulence, 312
Paratido-stomatitis, in calves, 142
Park, Bohan: Demonstration of *Shigella flexneri* antigens by means of hemagglutination test, 82
Pasteurella pestis, Studies on, in various flea species, 103, 107
Pérez N., José L.: Pathological physiology of salmonellosis.
II. Plasma cholinesterase in infections due to Enterobacteriaceae, 1
Pneumonitis, The activity of tetracycline on feline pneumonitis virus infection of chick embryos, 177
The effect of penicillin on multiplying and nonmultiplying populations of sensitive and resistant strains of feline pneumonitis virus, 229
Poliomyelitis, The preparation of poliomyelitis complement-fixing antigens from infected tissue culture fluids, 27
Pollack, Abou D.: The comparative pathology of Venezuelan equine encephalomyelitis, 55

Rabies, Experimental infection in insectivorous bats, 10
Randall, Elizabeth: Hemolysis and hemagglutination by normal and immune serums of erythrocytes treated with a nonspecies specific bacterial substance, 21
Rantz, Lowell A.: Hemolysis and hemagglutination by normal and immune serums of erythrocytes treated with a nonspecies specific bacterial substance, 211
Roberts, R. H.: Potential insect vectors of vesicular stomatitis in Wisconsin, 121
Rosen, H.: Studies on the biology of *Cryptococcus.*
VI. Amino acid composition of the somatic proteins of *Cryptococcus neoformans,* 208
Ross, Martin A.: Epizootic *Salmonella enteritidis* infection causing septic pulmonary phlebothrombosis in hamsters, 133
Ruda, Jacqueline: The effect of penicillin on multiplying and nonmultiplying populations of sensitive and resistant strains of feline pneumonitis virus, 229

Sadun, E. H.: The flocculation test with a purified antigen in the diagnosis of trichinosis in humans, 172
Salmonella enteritidis, Epizootic *S. enteritidis* infection causing septic pulmonary phlebothrombosis in hamsters, 133
Salmonellosis, pathological physiology of, 1
Scheff, George J.: Concerning the mechanism of bartonella anemia in splenectomized rats, 113
Scheff, Irene M.: Concerning the mechanism of bartonella anemia in splenectomized rats, 113
Schjerning-Thiesen, K.: Detection of complement fixing antibodies against foot-and-mouth disease in cattle serum, 266
Schur, Eva M.: Dynamics of bacterial infections in mice under conditions known to alter survival time, 198
Shigella flexneri, Demonstration of *Sh. flexneri* antigens by means of hemagglutination test, 82
Shimada, Tomiko: Studies on *Pasteurella pestis* in various flea species.
I. Infection of flea species with avirulent strains of *Past. pestis,* 103
II. Simplified method for the experimental infection of fleas, 107
Smith, C. E.: Relationship of the in vivo form of *Coccidioides immitis* to virulence, 312
Smith, Dorothy G.: The comparative pathology of Venezuelan equine encephalomyelitis, 55
Snellbaker, Leroy F.: Effects of animal viruses on host cell metabolism.
I. Effects of encephalomyocarditis virus on nucleic acids and phosphorus fractions of Ehrlich ascites tumor cells, 270
Stamm, D. D.: Experimental rabies infection in insectivorous bats, 10
Sutton, R. M.: The pathogenic components of an experimental fusospirochetal infection, 15
Suyemoto, W.: Studies on *Pasteurella pestis* in various flea species.
I. Infection of flea species with avirulent strains of *Past. pestis,* 103
II. Simplified method for the experimental infection of fleas, 107

Taliaferro, William H.: The restoration of hemolysin-forming capacity of X-irradiated rabbits by tissue and yeast preparations, 75
The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums, 193
Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation, 300
Talmage, David W.: The blocking of labeled antibody uptake as a measure of the relative combining capacity of anti-red cell serums, 277
The effect of repeated injections of sheep red cells on the hemolytic and combining capacities of rabbit antiserums, 293
Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation, 300
Tatum, Arthur L.: Effects of hypoxia on rats infected by *Trypanosoma lewisi,* 127
Thorbecke, G. J.: Antibody and γ globulin formation in vitro in hemopoietic organs, 157
Tissue cultures, The effect of thyroxin on respiration and virus growth in chick embryo tissue cultures, 239
Toxoplasmosis, Serological evidence of, in animals, 52
Trichinella spiralis, Some effects of cortisone in mice with acquired immunity to, 39
Cellular factors in acquired immunity to, as indicated by cortisone treatment of mice, 187
Trichinosis, The flocculation test with a purified antigen in the diagnosis of, in humans, 172
Trypanosoma lewisi, Effects of hypoxia on rats infected by, 127
Tularemia, Experimental studies on argasid ticks as possible vectors of tularemia, 67

Usman, L. L.: Studies on the biology of *Cryptococcus.*
VI. Amino acid composition of the somatic proteins of *Cryptococcus neoformans,* 208

Van der Hoeden, J.: Leptospirosis canicolaris in pigs and its probable transfer to human beings, 33
Vanella, J. M.: Transmission studies with encephalomyocarditis virus, 98
Vesicular stomatitis, Potential insect vectors of, in Wisconsin, 121
Victor, Joseph: The comparative pathology of Venezuelan equine encephalomyelitis, 55

Wheeler, C. M.: Studies on *Pasteurella pestis* in various flea species.
I. Infection of flea species with avirulent strains of *Past. pestis,* 103
II. Simplified method for the experimental infection of fleas, 107
Winter, W. D., Jr.: Enhancement of candida infection: differential distribution of renal lesions in mice treated with aureomycin, 150
Wilson, Catherine: Epizootic *Salmonella enteritidis* infection causing septic pulmonary phlebothrombosis in hamsters, 133

X irradiation, The restoration of hemolysin-forming capacity of X-irradiated rabbits by tissue and yeast preparations, 75

Yamakawa, Yoko: Studies on *Pasteurella pestis* in various flea species.
I. Infection of flea species with avirulent strains of *Past. pestis,* 103
II. Simplified method for the experimental infection of fleas, 107

Zebovitz, Marian M.: The effect of penicillin on multiplying and nonmultiplying populations of sensitive and resistant strains of feline pneumonitis virus, 229
Zuckerman, Anne: Hemolysis and hemagglutination by normal and immune serums of erythrocytes treated with a nonspecies specific bacterial substance, 211